# HAEMATOPOIETIC STEM CELL RESPONSE IN ALCOHOL INDUCED LIVER INJURY

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**Doctor of Medicine**

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**2009**

*To my wife Wendy and daughter Zoë*

# Declaration

I declare that the work contained in this thesis is composed by me. All studies were performed in the University of Edinburgh's Department of Hepatology and the Liver Unit, in the Royal Infirmary of Edinburgh, Scotland. I have not submitted the thesis for any other degree, postgraduate diploma or professional qualification.

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# Contents

## Acknowledgements

I wish to thank Dr John N Plevris for his support, enthusiasm and supervision of all the studies. I am also indebted to Dr Philip N Newsome for his expertise and guidance on the stem cell studies. I am most grateful to Ms Shelagh Boyle and Professor Wendy A Bickmore for their expert guidance with the fluorescent *in situ* hybridisation techniques (FISH) and Ms Shonna McCall for her expert assistance with flow cytometry and cell sorting. I would like to thank Professor David J Harrison for his expertise in liver histopathology and Professor Peter C Hayes for his support and advice with the stem cell studies.

I would like to extend my gratitude to Mrs Anne Pryde and Mrs Patricia Lee from the Hepatology Laboratory for their technical assistance with blood sample processing, immunohistochemistry and serum enzyme linked immunosorbent assays. I wish to thank Ms Rachael Brown for her assistance with the immunohistochemical analysis for hepatic stem cells and Ms Karen O'Neil and Mr Michael Hart for their assistance in serum chemokine analysis. I also wish to thank Dr Qing Liu for her assistance with peripheral blood sample collection and analysis.

Most important of all, I wish to thank my wife for her assistance with the diagrams and illustrations as well as her support, encouragement and patience. A special thanks also to my parents and brothers for their encouragement and support.

# Abstract of Thesis


**Background:**

Haematopoietic stem cell (HSC) contribution to liver repair varies considerably with some of the observed variation and putative mechanisms (transdifferentiation and fusion) possibly related to the type and severity of liver injury. More recently it has been recognised that HSCs may also contribute to liver fibrosis. In both rodents and humans, ethanol exposure induces hepatocyte death and inhibits the proliferative response of adult hepatocytes resulting in the accumulation of hepatic stem cells within the liver as part of a regenerative response to injury, although the precise origin of these cells (hepatic *vs.* BM-derived) remains unclear. Studies on the extent of peripheral blood HSC mobilisation in acute or chronic liver injury is however lacking and the degree of contribution to liver repair from mobilised HSCs remains uncertain.

**Aims:**

1) To investigate the mobilisation and hepatic recruitment of HSCs in patients with alcohol induced liver injury and define their contribution to parenchymal and non parenchymal liver cell lineages.
2) To establish that mobilised HSCs in alcohol induced liver injury are functional and demonstrate pluripotent stem cell properties.
3) To study the role of inflammatory cytokines and chemokine axes in regulating the mobilisation and hepatic recruitment of HSCs in alcohol induced liver injury.

**Methods:**

Liver biopsies from alcoholic hepatitis (AH) patients and male patients who had received a female liver transplant (and who subsequently developed AH) were analysed for HSC content using immunohistochemical and fluorescent *in situ* hybridisation techniques

(FISH). FISH for Y-chromosome was performed on liver tissue, along with co-staining for hepatocyte, biliary, myofibroblast and hepatic parenchymal cells proliferation markers. Peripheral blood HSC ($CD34^+$) levels were quantified in AH patients using flow cytometry and $CD34^+/CD45^+$ HSC were collected and cultured in colony forming unit (CFU) assays. CXCR3/CXCR4 receptor cell expression on mobilised $CD34^+$ HSCs were quantified in AH patients using flow cytometry. Serum SDF-1, MMP-9, G-CSF, IL-8, IP-10, MIG, RANTES, MCP-1 and neutrophil elastase (NE) levels in AH patients were measured by enzyme linked immunosorbent assay (ELISA). Hepatic expression of SDF-1, MMP-9 and NE were analysed using immunohistochemical techniques in AH liver biopsies.

**Results:**

Patients with AH had increased numbers of $CD34^+$ cells in liver tissue ($1.83 \pm 0.61\%$; $p<0.05$) and in blood ($0.2 \pm 0.06\%$; $p<0.05$) as compared with appropriately matched controls. A proportion of hepatic myofibroblasts were bone marrow (BM) derived (7.9% - 26.8%) as deemed by the co-localisation of the Y-chromosome and α-SMA staining. In the cross sex liver grafts with AH, 5.03% of the myofibroblasts were co-staining for CD34, suggesting that a population of $CD34^+$ cells were contributing to the hepatic myofibroblast population. In contrast, there was no evidence of BM contribution to hepatocyte or biliary cell differentiation, nor evidence of increased hepatocyte cell regeneration. Mobilised $CD34^+$ cell CFU frequency in AH patients were significantly higher as compared with NC HSCs ($154 \pm 38$ *vs.* $44.75 \pm 28.4$, $p<0.05$). There was no significant difference in either the percentage of $CD34^+$ expressing CXCR4/CXCR3 or receptor density / cell in the AH group when compared with the NC. Patients with AH had significantly increased serum SDF-1 ($1.4 \pm 0.35$ng/mL, $p<0.05$) levels and reduced hepatic SDF-1 expression as compared with NC. Serum MMP-9 ($260.05 \pm 70.11$ng/mL, $p<0.05$) levels were significantly increased whilst hepatic expression of MMP-9 was unchanged in AH patients as compared with NC. Serum G-CSF levels ($29.73 \pm$

10.1pg/mL, $p<0.05$) were significantly increased in AH patients as compared with NC and there was a positive correlation between serum G-CSF levels and circulating $CD34^+$ cell levels in patients with AH. Serum IL-8, IP-10, MIG RANTES, MCP-1 and NE levels demonstrated a non specific response to alcohol induced liver injury.

**Conclusions:**

Alcohol induced liver injury mobilises $CD34^+$ HSCs into the peripheral circulation and recruits them into the liver. These cells contribute to the hepatic myofibroblast population but not to parenchymal lineages and their presence within the liver does not promote hepatocyte repair. Mobilised HSCs from AH patients were functional and displayed true stem cell potential at a level higher than control HSCs. Serum SDF-1, MMP-9 and G-CSF rather than chemokine receptor expression, plays a central role in regulating the mobilisation of $CD34^+$ stem cells in alcohol induced liver injury.

# Aims and Structure

This thesis aims to investigate the mobilisation and hepatic recruitment of haematopoietic stem cells in patients with alcohol induced liver injury and define their contribution to parenchymal and non parenchymal liver cell lineages. It aims to give insights into the role inflammatory cytokines and chemokines regulate the process of haematopoietic stem cell mobilisation and hepatic recruitment in alcohol induced liver injury.

The introduction of this thesis **(Chapter 1)** provides a general background on the multilineage plasticity of haematopoietic stem cells and the mechanisms involved in epithelial lineage regeneration. The contribution of haematopoietic stem cells to liver repair is described in detail, as well as the contribution of bone marrow derived stem cells to liver fibrosis. Current literature on the mobilisation of peripheral blood haematopoietic stem cells and the regulatory role of inflammatory cytokines and chemokine axes in liver injury is analysed. A discussion of the hepatic recruitment of haematopoietic stem cells in liver injury together with a description of the pathogenesis of alcohol induced liver injury and the hepatic progenitor cell response in alcohol induced liver injury is included. The application of bone marrow stem cell therapy in liver disease and a synopsis of published clinical trials in this area is also discussed. The concluding section provides explanation as to why alcohol related liver injury was identified as a suitable injury model to study HSC mobilisation and recruitment as well as to provide a discussion of the unanswered questions of HSC mobilisation and contribution to liver repair in acute alcohol induced liver injury that will be subsequently addressed in the thesis.

The first experimental chapter **(Chapter 2)** demonstrates the accumulation and expansion of progenitor stem cells which express haematopoietic stem cell markers in

alcohol induced liver injury and discusses whether hepatic progenitor cells are from an endogenous hepatic or bone marrow derived source. The next chapter **(Chapter 3)** demonstrates the hepatic recruitment of bone marrow derived stem cells in alcohol induced liver injury and explores the contribution of bone marrow derived stem cells to parenchymal and non parenchymal liver cell lineages. The promotion of endogenous hepatocyte proliferation in a paracrine fashion by haematopoietic stem cells in alcohol induced liver injury is also investigated. The mobilisation of endogenous haematopoietic stem cells into the peripheral circulation of patients with alcohol induced liver injury is described in the following chapter **(Chapter 4)**. The stem cell potential and chemokine receptor profiles of these mobilised haematopoietic stem cells are evaluated and their contribution to regulating this mobilisation process in alcohol induced liver injury is described.

The final analytical work **(Chapter 5)** describes the role of inflammatory cytokines and chemokine axes in the mobilisation and recruitment of haematopoietic stem cells in alcohol induced liver injury.

Finally there is a detailed critical discussion of the research highlighting the main results and insights made by this thesis **(Chapter 6)**.

# Abbreviations

| | |
|---|---|
| AAC | Abstinent alcoholic cirrhosis |
| 2-AAF | 2-Acetylaminofluorene |
| AH | Alcoholic hepatitis |
| α-SMA | α-Smooth muscle actin |
| ANOVA | Analysis of variance |
| BM | Bone marrow |
| $CCl_4$ | Carbon tetrachloride |
| CFU | Colony forming unit |
| CT | Computerised tomography |
| CXCR | CXC chemokine receptor |
| DAB | Diaminobenzidine |
| DAPI | 4, 6-diaminidino-2-phenylindole |
| DF | Discriminant function - Maddrey's |
| ELISA | Enzyme linked immunosorbent assay |
| ESC | Embryonic stem cell |
| FISH | Fluorescent *in situ* hybridisation |
| FITC | Fluorescein isothiocynate |
| FAK | Focal adhesion kinase |
| FFPE | Formalin fixed paraffin embedded |
| FAH | Fumarylacetoacetate hydrolase |
| G-CSF | Granulocyte colony stimulating factor |
| HSC | Haematopoietic stem cell |
| HOC | Hepatic oval cell |
| HPC | Hepatic progenitor cell |
| HpSC | Hepatic stellate cells |

| | |
|---|---|
| HCV | Hepatitis C virus |
| HGF | Hepatocyte growth factor |
| HRP | Horseradish peroxidase |
| IP-10 | Interferon-γ-induced protein-10 |
| IL-8 | Interleukin-8 |
| ISHAGE | International Society for Haemotherapy and Graft Engineering |
| IMDM | Iscoves modified dulbecco's media |
| MMP-9 | Matrix metalloproteinase-9 |
| MSC | Mesenchymal stem cell |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MIG | Monokine-induced by interferon-γ |
| MNCC | Mononuclear cell count |
| NE | Neutrophil elastase |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NOD/SCID | Non-obese diabetic/severe combined immunodeficient |
| NC | Normal controls |
| OLT | Orthotopic liver transplantation |
| PH | Partial hepatectomy |
| PBS | Phosphate buffered saline |
| PE | Phycoerythrin |
| PBC | Primary biliary cirrhosis |
| PKC | Protein kinase C |
| PT | Prothrombin time |
| RANTES | Regulated on T cell activation, normal T cell expressed and secreted |
| RPE-CY5 | R-phycoerythrin-cyanine dye 5 |
| SEM | Standard error of the mean |
| SCF | Stem cell factor |
| SDF-1 | Stromal cell-derived factor-1 |

| | |
|---|---|
| TAA | Thioacetamide |
| TGF-ß | Transforming growth factor-beta |
| TIMPs | Tissue inhibitors of metalloproteinases |
| TNF-α | Tumour necrosis factor alpha |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VLA-4 | Very late antigen-4 |

# Chemokine Nomenclature Appendix

| Old nomenclature | New nomenclature* |
|---|---|
| IP-10 | CXCL10 |
| IL-8 | CXCL8 |
| MCP-1 | CCL2 |
| MIG | CXCL9 |
| MIP-2 | CXCL2 |
| RANTES | CCL5 |
| SDF-1 | CXCL12 |

The chemokines in this thesis have been referred to in the old nomenclature style as this form of nomenclature was still widely used and referred to in publications at the time of conducting the experiments for this thesis.

*As proposed by the International Union of Immunological Societies/World Health Organisation Subcommittee on chemokine nomenclature (IUIS/WHO) 2001.

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# CHAPTER 1

# Introduction

## 1.1 Haematopoietic stem cell multi-lineage plasticity

The bone marrow (BM) compartment in humans comprises of committed progenitor cells, mesenchymal stem cells (MSC) (which form stromal cells and cells of mesodermal origin) and a larger stem cell population called haematopoietic stem cells (HSCs) (Petersen et al. 1999). These HSCs have long been known to possess the unique capacity for self-renewal and differentiation into cell lineages which sustain the formation of the blood and immune systems throughout life. More recently it has been observed that this plasticity extends to non-haematopoietic lineages such as, hepatic progenitor cells, hepatocytes, cholangiocytes (Alison et al. 2000; Petersen et al. 1999; Theise et al. 2000b), skeletal muscle cells (Ferrari et al. 1998), cardiac myocytes (Orlic et al. 2001) neurons (Li et al. 2001), epithelial cells of the lung, GI tract and skin (Korbling et al. 2002). This pathway of differentiation can be bi-directional with reports demonstrating that both muscle and neuronal stem cells can also contribute to haematopoiesis (Bjornson et al. 1999; Jackson, Mi & Goodell 1999).

Furthermore, emerging data in the field of cardiac regeneration suggests that incoming stem cells can also contribute to tissue repair by promoting neoangiogenesis and minimising cardiomyocyte apoptosis (Jackson et al. 2001). HSCs may also play a role in stimulating endogenous hepatocyte proliferation in a paracrine fashion as evidenced by their contribution in rodent (Yannaki et al. 2005) and human studies (Gaia et al. 2006) in which HSC are mobilised with granulocyte colony stimulating factor (G-SCF).

This relatively new observation of HSC multilineage plasticity has raised hopes that such cells could, in the future, be utilised for the regeneration and reconstitution of damaged organ tissue. Embryonic stem cells (ESC) derived from the early post implantation embryo are a source of pluripotent stem cells which can be utilised in regenerative medical therapies however there are significant ethical and legal issues

associated with its use, thus making HSCs an attractive alternative source of stem cells. This process of epithelial lineage regeneration appears to occur via a process of either spontaneous cell fusion and/or transdifferentiation (Masson et al. 2004) and whatever the underlying mechanism by which the HSCs participate in tissue regeneration, it will still require the presence of HSCs to mobilise from the BM and to reach their target organ (Dalakas et al. 2005).

## 1.2 Adult stem cells and liver regeneration

Whilst the liver is a mitotically quiescent organ in adult humans and animals (Alison, Poulsom & Forbes 2001), hepatocytes have a remarkable capacity to meet the replacement demands during cellular loss (Alison 1998; Thorgeirsson 1996). Hepatocytes are numerous and respond to liver injury such as partial hepatectomy and centrilobular injury by one or two cell cycles although individual hepatocytes have the capacity for up to 70 cell cycle divisions as demonstrated in serial transplantation experiments (Overturf et al. 1997). However, when either chronic or extensive damage is inflicted upon the liver or when hepatocyte proliferation is inhibited, a facultative cellular compartment of ductular progenitor cells, located within the smallest branches of the intrahepatic biliary tree is activated and leads to liver repair (Sell 2001; Thorgeirsson 1996). In rodents these cells are named hepatic oval cells (HOC) whilst in human they are referred to as hepatic progenitor cells (HPCs) (Roskams et al. 2004).

More recently, several groups have demonstrated that BM derived HSCs may contribute to liver repair (Alison et al. 2000;Kollet et al. 2003;Lagasse et al. 2000;Petersen et al. 1999;Theise et al. 2000a;Theise et al. 2000b). The contribution of HSCs to liver repair has varied, but is generally related to the presence and severity of liver injury. These haematogenously-derived stem cells have a very long proliferative potential and respond to injury when neither HPCs nor mature hepatocytes are able to restore the injured liver tissue (Sell 2001). Thus the restitutive response of the liver to different injuries

has been proposed to include three levels of proliferating cells; 1) the hepatocyte, 2) the endogenous ductular progenitor cell or HOC and 3) a pluripotent stem cell derived from circulating BM cells (Alison 1998).

Controversy has recently arisen as to whether HSCs contribute to the hepatocyte lineage in liver injury via transdifferentiation alone or by adopting the phenotype of hepatocytes after spontaneous cell fusion (Austin & Lagasse 2003). Reports in favour of the fusion hypothesis have demonstrated that stem cells can adopt the phenotype of other cell lines by fusing with embryonal stem cells (Terada et al. 2002; Ying et al. 2002). Terada et al demonstrated that murine bone marrow cells can fuse spontaneously with embryonic stem cells when co-cultured *in vitro* with interleukin-3 and adopting the phenotype of the recipient embryonic stem cell. Furthermore, Wang et al demonstrated that murine BM derived hepatocytes are generated by *in vivo* cell fusion between donor BM cells and host hepatocytes in fumarylacetoacetate hydrolase FAH-/- deficient mice (Wang et al. 2003c). In another murine injury model, Kashofer et al transplanted human-enriched HSCs into carbon tetrachloride ($CCl_4$) induced liver damaged non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice and identified donor derived hepatocytes. These hepatocytes occurred through cell fusion and because no selective pressure was applied to retain the human genomic material, it was lost over time, leading to a variable phenotype of the chimeric cells (Kashofer, Siapati, & Bonnet 2006). In support of transdifferentiation, several groups have demonstrated that HSCs can differentiate into hepatocytes (Ishikawa et al. 2003; Newsome et al. 2003) and pancreatic endocrine cells (Ianus et al. 2003) without any evidence of cell fusion. Recently Oh et al demonstrated that BM derived hepatic oval/ progenitor cells could differentiate into hepatocytes in a murine 2-acetylaminofluorene (2-AAF) / partial hepatectomy (PH) liver injury model without any evidence that fusion was contributing to the differentiation of donor-derived oval cells. The mechanism of HSC hepatic regeneration remains unresolved and clearly any future stem cell research will have to distinguish HSC transdifferentiation from fusion events (Oh et al. 2007).

Whatever the mechanism of hepatic regeneration is, the trafficking of HSCs to the liver will play an important component of the reparative process in liver injury.

The contribution of HSCs to hepatocyte lineages in both rodents and humans remains a controversial area with data both supporting (Alison et al. 2000; Lagasse et al. 2000; Petersen et al. 1999; Theise et al. 2000a; Theise et al. 2000b; Wang et al. 2003b) and rebutting findings (Dahlke et al. 2003; Kanazawa & Verma 2003; Wu et al. 2003). This may, in part, reflect the types of cells used, the injury models used and the methods used to detect stem cell progeny. Nevertheless a therapeutic role of HSCs in liver injury has been described in rodents (Lagasse et al. 2000; Sakaida et al. 2004) albeit with varying contributions of transdifferentiation and fusion. Legasse et al (Lagasse et al. 2000) demonstrated the ability of BM derived cells to correct a metabolic liver disease in female mice deficient in the enzyme fumarylacetoacetate hydrolase FAH-/- an animal model of fatal hereditary tyrosinaemia type 1. The mice that received intravenous injections of highly purified adult HSCs were rescued and biomedical restoration of liver function was demonstrated.

In other models, and in particular in humans, the contribution that HSCs make to liver repair by transdifferentiation is lower, in the order of 0.011-20% (Alison et al. 2000;Newsome et al. 2003;Theise et al. 2000b;Wu et al. 2003). Both Theise et al (Theise et al. 2000b) and Alison et al (Alison et al. 2000) have demonstrated that BM cells can contribute to liver regeneration in damaged human liver tissue, although the degree of hepatic engraftment was highly variable. In both studies liver specimens from human recipients of therapeutic BM or liver transplants, in which there was gender discordance between donor and recipient, were analysed by *in situ* hybridisation for the Y-chromosome. Alison et al reported that the frequency of Y positive hepatocytes ranged from 0.5 to 2%, a value that correlates with previous murine studies whilst Theise et al reported a more variable hepatocyte engraftment range. The variability

in engraftment rate between the two studies can partly be due to Theise et al using a five fold correcting factor to account for the frequency of Y-chromosome positive hepatocytes in the normal male liver. Theise et al also noted that the extent of hepatocyte engraftment correlated with the extent of liver injury (Theise et al. 2000b). With mild liver injury, hepatocyte engraftment occurred at lower levels ranging from 5% to 19% and in one case of severe injury (recurrent hepatitis C), the engraftment rate increased to 40%. Further studies have however reported significantly lower levels of HSC contributions to liver repair. To improve on this level of contribution will require a greater understanding of the mechanisms by which stem cells mobilise from the BM and home to injured organs. There remains a pressing need however, for further studies to confirm or refute the claims that stem cells can lead to improved liver repair and hence survival in either rodent or human setting.

## 1.3 Bone marrow stem cells and liver fibrosis

Chronic hepatic injury is usually accompanied by progressive fibrosis and the inflammatory response to the hepatic injury activates hepatic stellate cells (HpSC) which proliferate and synthesise collagen (Kallis, Alison, & Forbes 2007). These HpSC display alpha-smooth muscle actin (α-SMA), a myofibroblasts marker and are thought to be central in the pathogenesis of liver fibrosis (Lau et al. 2005; Nouchi et al. 1991; Russo et al. 2005). Recently it has been recognised that BM derived stem cells contribute more significantly to non parenchymal cell lineages such as hepatic endothelium (Gao, McAlister, & Williams 2001), stellate cells (Baba et al. 2004) and myofibroblasts (Russo et al. 2006). Russo et al demonstrated that in chronic chemical injury up to 70% of the murine hepatic myofibroblast population were derived from the mesenchymal stem cell (MSC) component of the BM (Russo et al. 2006). Furthermore, studies examining BM transplantation from the Colla mouse (which produces a mutated collagenase resistant form of collagen) into wild-type mice demonstrated that the phenotype of liver fibrosis would appear, in part, to be

dependent on the BM (Russo et al. 2006). Recently, another cell type was implicated with the generation of liver fibrosis. Kisseleva et al demonstrated that BM-derived fibrocytes contributed up to 5 to 10% of all collagen expressing cells in the bile duct ligated model of injury (Kisseleva et al. 2006). Fibrocytes are circulating cells derived from haematopoietic stem cells (Ebihara et al. 2006) that express markers for both haematopoietic cells and fibroblasts, producing collagen and other matrix proteins, but which generally do not express α-SMA (myofibroblast marker) *in vivo* (Abe et al. 2001). In addition to their contribution to hepatic scarring they have been shown to make a significant contribution to pulmonary (Phillips et al. 2004), renal (Sakai et al. 2006) and cutaneous (Mori et al. 2005) scarring. Analysis of archival human tissue has identified that 6-22% of hepatic myofibroblasts are BM derived (Forbes et al. 2004).

## 1.4 HSC mobilisation in liver injury

Human studies have demonstrated increased levels of circulating HSCs in response to a systemic injury such as acute sickle cell crisis, ischaemic stroke, acute myocardial infarction and surgical trauma (Grzelak et al. 1998; Lamming et al. 2003; Paczkowska et al. 2005). In the context of human liver injury, elevated levels of peripheral blood HSCs have been demonstrated following extensive liver resection in patients with malignant, primary or secondary liver diseases (De Silvestro et al. 2004). Furthermore, following partial hepatectomy in living liver donors there appears to be a mobilisation of BM derived progenitor cells with *in-vitro* hepatic differentiation potential (Gehling et al. 2005). Kyriakou et al demonstrated elevated levels of circulating peripheral blood HSCs in patients with autoimmune hepatitis but not in patients with primary biliary cirrhosis or cirrhotics of viral or alcohol aetiology (Kyriakou et al. 2003). A recent study by Lemoli et al demonstrated HSCs mobilisation to be more pronounced in liver transplant patients with ischaemic/reperfusion injuries as compared with patients undergoing liver resections and suggesting that the greater the extent of liver tissue injury the higher the degree of HSC mobilisation (Lemoli et al. 2006). Studies on the

extent of peripheral blood HSC mobilisation in acute or chronic alcohol liver injury is however lacking and the degree of contribution to liver repair from mobilised HSCs remains uncertain.

### 1.4.1 SDF-1 and HSC mobilisation from the bone marrow

In the adult BM, the release of HSCs into the peripheral circulation is regulated in part by the CXC chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 (Aiuti et al. 1997; Kim & Broxmeyer 1998a; Lapidot & Petit 2002; Petit et al. 2002). SDF-1 is a potent chemoattractant for HSCs and is produced by various BM stromal cell types and epithelial cells in a broad range of normal tissues including the liver (Bajetto et al. 1999; Casamayor-Palleja et al. 2001; Godiska et al. 1995; Jiang et al. 1994; Pablos et al. 1999; Tashiro et al. 1993; Zaitseva et al. 2002) ***(see table 1)***. It plays a major role in the homing, migration, proliferation, differentiation and survival of many cell types including human and murine haematopoietic stem/progenitor cells (Aiuti et al. 1997; Bleul, Schultze, & Springer 1998; D'Apuzzo et al. 1997; Lapidot & Petit 2002; Mohle et al. 2001; Petit et al. 2002; Whetton & Graham 1999). Knockout mice deficient in SDF-1 exhibit disturbed haematopoiesis, and knockout mice deficient in the CXCR4 receptor die in utero (Kawabata et al. 1999; Nagasawa et al. 1996), underlining their importance.

SDF-1 is highly conserved between mice and humans (Kollet et al. 2002; Sweeney & Papayannopoulou 2001), mediating its effect through the CXCR4 receptor that is expressed on $CD34^+$ HSCs, mononuclear leucocytes as well as a variety of stromal cells (Kollet et al. 2002). CXCR4 is a G-protein-coupled, 7-transmembrane receptor and is the only known receptor for SDF-1 (Nagasawa, Tachibana, & Kawabata 1999). The interaction between SDF-1 and CXCR4 has been demonstrated to trigger multiple intracellular signals including calcium mobilisation and phosphorylation of adhesion components such as extracellular-signal regulated kinases 1 and 2 (ERK-1 and -2),

proline-rich tyrosine kinase 2 (Pyk-2), focal adhesion kinase (FAK) and protein kinase C (PKC) (Roland et al. 2003;Wang, Park, & Groopman 2000). In the adult BM, the release of HSC into the peripheral circulation is controlled in part by a concentration gradient of SDF-1 established within the BM microenvironment (Bleul et al. 1996;Kim et al. 1998b;Kim & Broxmeyer 1998a). Reduction of BM SDF-1 levels has been shown to result in release of HSC into the peripheral circulation, an effect partly mediated by granulocyte colony-stimulating factor (G-CSF) which induces the release and synthesis of neutrophil proteases such as neutrophil elastase (NE), cathepsin G and matrix metalloproteinases (MMP) (Petit et al. 2002). In addition, increased expression of SDF-1 in the peripheral circulation facilitates further mobilisation of HSCs down a concentration gradient (Hattori et al. 2001).

In the context of human liver injury there are several reports demonstrating increased circulating plasma levels of SDF-1 in autoimmune and viral diseases, in conjunction with increased expression of SDF-1 in the parenchyma of rejecting liver transplants and viral/autoimmune liver diseases (Goddard et al. 2001;Terada et al. 2003). These observations have also been reported in murine liver injury models (Hatch et al. 2002;Kollet et al. 2003), suggesting that liver injury may by the expression of SDF-1, produce a concentration gradient between liver and BM, which in turn facilitates the recruitment of inflammatory cells and HSCs from the BM into the circulation and then into the liver (Dalakas et al. 2005;Kollet et al. 2003;Terada et al. 2003) ***(see figure 1a-1c)***.

The mechanism by which SDF-1 influences HSC mobilisation is unclear, although it is thought to involve specific changes to the adhesion of progenitor cells to the BM microenvironment via the modulation of adhesion molecules such as the integrin-dependant very late antigen-4 (VLA-4) (Peled et al. 2000). *In vitro* there is an increased trans-endothelial migration of human progenitor cells towards a gradient of SDF-1

(Mohle et al. 2001;Netelenbos et al. 2002) and furthermore SDF-1 has been shown to promote the survival of circulating $CD34^+$ HSCs by counteracting apoptosis via the activation of the phosphotidyl inositol 3 kinase (PI3-K)/Akt pathway (Lataillade et al. 2002).

It has been speculated that the release of proteolytic enzymes and chemokines from injured liver into the circulation could also facilitate mobilisation and recruitment of HSCs (Kollet et al. 2003). Studies with G-CSF have revealed neutrophil proteolytic enzymes such as neutrophil elastase (NE), cathepsin G and MMP's, including MMP-2 and MMP-9, resulting in the proteolytic degradation of SDF-1 in the BM, thus facilitating the release of stem cells (Levesque et al. 2003;Petit et al. 2002). NE and cathepsin-G are capable of proteolytic cleavage of key molecules vascular cell adhesion molecule-1 (VCAM-1) (Levesque et al. 2001) and SDF-1 (Petit et al. 2002), which regulate HSC trafficking in the BM. NE and CG deficient mice however display normal G-CSF induced HSC mobilisation (Levesque et al. 2004) and consequently the role of NE and cathepsin-G in HSC mobilisation remains uncertain.

### 1.4.2 MMP-9 and HSC mobilisation from the bone marrow

MMPs degrade extracellular matrix proteins and are known to play important roles in tissue inflammation, tumour growth and organ remodelling (Birkedal-Hansen et al. 1993;Visse & Nagase 2003). MMPs are secreted as zymogens (pro-MMPs) which are activated by a variety of proteinases and are inhibited by tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin (Birkedal-Hansen et al. 1993). In humans, MMP-9 is produced in a wide variety of cells types such as neutrophils, progenitor cells, endothelial cells, fibroblasts, connective tissue cells, tumour cells and parenchymal cells, including the liver (Birkedal-Hansen et al. 1993;Geisler et al. 1997). Human and animal studies have demonstrated that MMP-9 promotes the release of progenitor cells from the BM into the circulation by; 1) inducing the release

of soluble Kit-ligand (sKitL) from BM stromal cells which accelerates the proliferation and migration of HSCs, 2) cleaving the interaction of adhesion molecules VLA-4/ VCAM-1 between stromal cells and HSCs in the BM and 3) enhancing the SDF-1 induced migration potential of HSCs across the subendothelial basement membrane (Aiuti et al. 1997;Hattori et al. 2002;Heissig et al. 2002;Janowska-Wieczorek et al. 2000). In addition, MMP-9 induced recruitment of HSCs may also occur via other mechanisms such as the shedding of membrane bound stem cell factor (SCF) and the secretion of MMP-9 by progenitor cells in response to SDF-1 stimulation (Heissig et al. 2002;Janowska-Wieczorek et al. 2000). MMP-9 has been demonstrated to have an active involvement in liver remodelling in cirrhosis and inflammation as well as regulating hepatocyte regeneration after partial hepatectomy (Haruyama et al. 2000;Kuyvenhoven et al. 2004b;Lichtinghagen et al. 2003).

Human studies have demonstrated elevated serum and plasma MMP-9 levels in various types of liver injury including acute allograft rejection (Kuyvenhoven et al. 2004b), ischaemic reperfusion injury (Kuyvenhoven et al. 2003;Kuyvenhoven et al. 2004a), chronic viral hepatitis (Chung et al. 2004;Leroy et al. 2004) and alcoholic liver cirrhosis (Kwon et al. 2003), suggesting that there is a correlation between disease severity/progression and MMP-9 expression. In these studies 70-80% of the serum and plasma MMP-9 measured, appeared in the active complex form and could be detected in serum samples from as early as 30 minutes and greater than 1 week after an acute injury process. In chronic liver diseases such as alcoholic cirrhosis persistently elevated plasma activities of MMP-9 have been demonstrated suggesting that its expression reflects a process of ongoing extracellular matrix remodelling (Kwon et al. 2003).

Carbon tetrachloride ($CCl_4$) induced liver injury studies in rat and NOD/SCID mice (in which bone marrow cells were seen to transdifferentiate into hepatocytes) demonstrated

an increased expression and activation of MMP-9 in the liver, suggesting that this factor could potentially be involved in the stress-induced recruitment of HSC from the BM to the injured liver (Knittel et al. 2000;Kollet et al. 2003). A recent study by Hanumegowda et al (Hanumegowda et al. 2003), has also demonstrated an increased activation of MMP-9 in the livers of rats with monocrotaline induced liver injury (which inhibits hepatocyte proliferation and promotes an HOC response). This increase in MMP-9 activity was produced from either the endothelial cells or from an activation or influx of inflammatory cells into the injured hepatic parenchyma (Hanumegowda et al. 2003). Furthermore in a study by Watanabe et al, mice were injected with anti-Fas antibody (Jo2) to induce an acute hepatitis, demonstrating that MMP-9 expression in the circulation was elevated and accompanied by the recruitment of HSCs from the BM into the circulation (Watanabe, Haruyama, & Akaike 2003) ***(see figure 1b)***.

### 1.4.3 G-CSF and HSC mobilisation from the bone marrow

Immune and inflammatory stimuli promote the production of the cytokine G-CSF which induces the mobilisation and proliferation of HSCs and haematopoietic progenitor cells (HPCs). G-CSF is a potent cytokine which is utilised extensively for the mobilisation of HPC in both clinical and research applications (Mohle & Kanz 2007) and induces a highly proteolytic microenvironment in the BM during the mobilisation process (Levesque et al. 2002). Disruption of the SDF-1/CXCR4 signalling pathway is a key step in G-CSF mediated HSC mobilisation resulting in a decrease in bone marrow SDF-1 levels (Levesque et al. 2003;Petit et al. 2002;Semerad et al. 2002) and inactivation of the CXCR4 receptors on HSCs (Levesque et al. 2003).

Human studies have demonstrated that mobilised $CD34^+$ cell levels in the peripheral circulation of patients with acute myocardial infarction significantly correlates with elevated levels of endogenous G-CSF (Leone et al. 2006) and that G-CSF administration results in improved myocardial perfusion after subacute ST-segment

elevation myocardial infarction undergoing late revascularisation (Engelmann et al. 2006). Murine chemical liver injury ($CCl_4$, thioacetamide (TAA) & 2-AAF) and hepatectomy studies have demonstrated that G-CSF administration improves survival rate and liver regeneration by; 1) promoting endogenous repair and proliferation of host hepatocytes, 2) mobilising HSCs into the injured liver to differentiate into hepatocytes by enhancing the endogenous hepatic oval cell reaction and 3) promoting *in vivo* cell fusion of HSCs with hepatocytes (Liu et al. 2006;Piscaglia et al. 2007;Quintana-Bustamante et al. 2006;Theocharis, Margeli, & Kittas 1999;Yannaki et al. 2005). In the context of human liver injury, serum G-CSF concentrations are elevated after hepatic resection and ischaemic reperfusion injuries with preservation of hepatic parenchymal cell function (Hanazaki et al. 2001). Lemoli et al has demonstrated that tissue damage after orthotopic liver transplantation (OLT) and liver resection induces increased serum G-CSF levels but only the ischaemic reperfusion injury associated with OLT results in the mobilisation of BM stem/progenitor cells (Lemoli et al. 2006).

### 1.4.4 Chemokines IL-8, MIG, IP-10, RANTES and MCP-1 in HSC mobilisation

The interactions between MMP-9 and other chemokines such as Interleukin-8 (IL-8) has also been demonstrated in mobilisation studies whereby MMP-9 is rapidly induced in neutrophils following exposure to (IL-8) and resulting in the release of HSCs into the peripheral circulation (Carion et al. 2003;Fibbe et al. 1999;Pruijt et al. 2002;Van Zee et al. 1992) ***(see figure 1b)***. Elevated IL-8 levels have been demonstrated in the circulation and hepatic parenchyma of many human liver conditions including alcoholic hepatitis, viral hepatitis, chronic alcoholic liver disease and acute graft-versus-host disease following liver transplantation (Hill, Marsano, & McClain 1993;Huang et al. 1996;Sheron et al. 1993;Shimoda et al. 1998;Tilg et al. 1992). Thus IL-8 which is a known neutrophil chemoattractant in liver disease, also has the potential to induce the release of HSCs into the peripheral circulation via an indirect mechanism requiring

the activation of circulating neutrophils and the release of MMP-9 (Pruijt et al. 2002). The chemokines Monokine-induced by Interferon-γ (MIG) and Interferon-γ-induced Protein-10 (IP-10) which mediate their effect through chemokine receptor CXCR3, are thought to be important in recruiting T lymphocytes into the injured liver as well as playing a crucial role in $CD34^+$ HSC adhesion (Jinquan et al. 2000;Nanji et al. 1999). IP-10 and MIG can induce phosphorylation of $CD34^+$ progenitors which leads to CXCR3 expression and induces chemotaxis and adhesion of $CD34^+$ haematopoietic progenitor cells (Jinquan et al. 2001). This would suggest that CXCR3, IP-10 and MIG may be important in the cytokine/chemokine environment for the mobilisation, homing and recruitment of haematopoietic progenitor cells (Jinquan et al. 2001). IP-10 has both an *in vitro* and *in vivo* ability to enhance neural progenitor cell migration (Honeth et al. 2006) and also has a hepatoregenerative effect in a mouse model of acute paracetamol liver injury (Bone-Larson et al. 2001). The chemotactic cytokine RANTES and Monocyte Chemoattractant Protein-1 (MCP-1) have been shown to be up-regulated in $CD34^+$ stem cells when stimulated by inflammatory cytokines (including TNF-alpha) and which may contribute to an active role for $CD34^+$ HSCs during inflammation and immunological events (Umland et al. 2004). Gene expression of MCP-1 is up-regulated in human $CD34^+$ progenitor cells when implanted in a hypoxic environment and has been shown to generate rapid tissue neovascularisation, possibly via recruitment of monocytes and/or macrophages (van der Strate et al. 2007). These chemotactic and inflammatory cytokines could therefore also be important in the recruitment and differentiation of HSCs in liver disease.

## 1.5 Hepatic recruitment of HSCs in liver injury *(see figure 1c)*

Kollet et al have recently demonstrated the key role that SDF-1/CXCR4 mediated signalling plays in the migration of human progenitors to the murine liver. Neutralisation of the CXCR4 receptor with an anti-CXCR4 antibody significantly inhibited the homing of human cord blood or mobilised peripheral blood $CD34^+$ stem cells to the liver of irradiated NOD/SCID mice. Furthermore, injection of human SDF-1 into the murine liver parenchyma further enhanced the hepatic migration of human stem cells. SDF-1 expression has been reported in a variety of liver and non-liver conditions such as liver allograft rejection (Goddard et al. 2001), viral and autoimmune liver diseases (Terada et al. 2003), ischaemic brain injury (Stumm et al. 2002), myocardial infarction (Pillarisetti & Gupta 2001), inflammatory skin conditions (Pablos et al. 1999) and BM injury induced by total body irradiation or chemotherapy (Ponomaryov et al. 2000). It is unclear if this expression is an attempt to recruit inflammatory cells or HSCs towards the damaged organ, or if it is indeed entirely unrelated.

SDF-1 expression in rejecting liver transplants and viral/autoimmune liver diseases was seen to be confined to the biliary epithelium and other non-parenchymal cells, thus promoting the retention of $CXCR4^+$ lymphocytes and possibly HSCs in the portal tracts (Goddard et al. 2001;Terada et al. 2003). Hatch et al (Hatch, Zheng, Jorgensen, & Petersen 2002) were able to demonstrate that SDF-1 protein was up-regulated in the membrane fraction of the whole liver lysates. Notably however, this was only the case in animals that had undergone HOC regeneration models [partial hepatectomy (PH) and 2-acetylaminofluorene (2AAF) or 2AAF and $CCl_4$]. Animals that had undergone non-oval cell regeneration models of PH, $CCl_4$ alone and 2-AAF alone did not produce SDF-1 protein. Immunohistochemistry on the oval cell regeneration model liver sections revealed increased expression of SDF-1 in the hepatocytes adjacent to the proliferating oval cells and positive CXCR4 staining on these oval cells. This data

argues for the defined production of SDF-1 in forms of liver injury which may be attempting to recruit HSCs to the reparative process.

The cytokine hepatocyte growth factor (HGF) which is produced in the non parenchymal peri-sinusoidal cells of the liver and induces hepatocyte proliferation may in addition to SDF-1 also be involved in the migration and differentiation of HSC into the injured liver (Kiss et al. 2001;Kollet et al. 2003). Increased expression of HGF has been demonstrated in $CCl_4$ induced liver injury and in rodent HOC regeneration models suggesting that it is involved in stem cell proliferation, migration and differentiation (Alison et al. 1993;Wang et al. 2003b). Kollet et al have demonstrated that following liver injury levels of HGF were increased and contributed to the recruitment of human $CD34^+$ stem cells to the injured liver by increasing the motility of human progenitors and in synergy with stem cell factor (SCF) potentiated both CXCR4 and SDF-1 induced directional migration (Kollet et al. 2003).

## 1.6 Pathogenesis of alcohol induced liver injury

### 1.6.1 Alcohol induced oxidative stress

Current evidence suggests that alcoholic liver damage involves all hepatic cellular components (hepatocytes, Kupffer, stellate and endothelial cells) at some stage of its pathogenesis and is the end result of a complex interplay between ethanol metabolism, inflammation and immunity (Sougioultzis et al. 2005). Ethanol is oxidized in hepatocytes first to acetaldehyde and subsequently to acetate, via two well-studied metabolic pathways. The first is the combination of cytosolic alcohol dehydrogenase and mitochondrial aldehyde dehydrogenase leading to its sequential oxidation; the second is the ethanol-inducible microsomal ethanol oxidizing system (MEOS) with its major component, cytochrome P450 2E1 (CYP2E1) (Tsukumato & Lu 2001). Ethanol oxidation in the liver parenchyma generates reactive free radicals such as

reactive oxygen (ROS) and nitrogen intermediates (RNS) leading to oxidative stress, a condition that is further reinforced by the depletion of the antioxidants glutathione and vitamin E observed in chronic alcoholics (Bjorneboe et al. 1988;Tsukamoto & Lu 2001). Oxidative stress in alcoholic liver disease is therefore mediated by both an increase in pro-oxidant overproduction and a decrease in antioxidant defences. Oxidative stress primes the transcription of several cytokines and growth factors, potentially from all hepatic cell types, leading to the attraction of immune cells to the hepatic parenchyma and resulting in the peroxidation of membrane and lipoprotein lipids which facilitate cellular necrosis, apoptosis and activation of fibrogenesis (Natori et al. 2001;Stewart, Jones, & Day 2001;Ziol et al. 2001).

Acetaldehyde and other reactive molecules generated through ethanol oxidation have the propensity to bind covalently to proteins to form adducts which are potential neo-antigens that trigger host's adaptive immune response. Anti-acetaldehyde antibodies have been found in alcoholics (Koskinas et al. 1992;Niemela et al. 1987) and correlated with the severity of liver injury (Viitala et al. 1997) suggesting that they are probably involved in alcohol induced hepatic damage. Oxidative stress may also facilitate the production of auto-antibodies against phospholipids, alcohol dehydrogenase, cytochrome P4502E1 and antinuclear antibodies, which are detectable in 25-50% of patients with AH and cirrhosis (Chedid et al. 1994;Laskin et al. 1990;Ma et al. 1997;Vidali et al. 2003). However, the role of autoimmunity in the pathogenesis of liver damage in alcoholics still remains speculative (Albano 2002).

Apart from the direct and indirect insults of ethanol to the liver, recent evidence suggests that alcohol ingestion also increases the translocation of endotoxin, and possibly other noxious substances, from the gut lumen to the portal circulation (Bode, Kugler & Bode 1987;Keshavarzian et al. 1999). Endotoxin in turn, stimulates Kupffer cells to produce cytokines and free radicals, therefore perpetuating the oxidative stress, increasing further the hepatic cytokine load. Taken together, all the above constitute

the current working hypothesis towards a rational and mechanistic approach to alcohol mediated liver injury. It should be pointed out that the exact sequence of events that leads to severe liver damage in alcoholics is still incompletely understood.

### 1.6.2 Cytokines and inflammatory mediators in alcohol liver injury

A lot of attention has been focused recently on the role of tumour necrosis factor alpha (TNF-α) and apoptosis in the pathogenesis of alcohol induced liver injury. TNF-α is a macrophage-derived factor and is considered a key mediator in a wide variety of biological processes such as fever, septic shock, tissue injury, tumor necrosis and apoptosis. TNF-α transcripts trimerize to form the 51kD active TNF-α ligand which can bind to either a 55kD receptor (TNFR1), present on most cell types, or to a 75kD receptor (TNFR2), expressed mainly by immune and endothelial cells (Aggarwal 2003). The wide distribution of TNF receptors provides a possible explanation for its diverse biological effects. After binding to its receptors, TNF-α triggers a complex intracellular signalling cascade involving the activation of the transcription factor NF-kB and mitogen-activated protein kinases (MAPKs), resulting in the production of proinflammatory cytokines, such as interleukin 6 (IL-6) and interleukin 8 (IL-8) and upregulation of adhesion molecules (Aggarwal 2003). TNF-α can also induce apoptosis in a wide variety of cell types (Gaur & Aggarwal 2003). However, normal hepatocytes seem to be resistant to the pro-apoptotic action of TNF-α, an effect that is thought to be mediated by the anti-apoptotic properties of NF-kB (Tilg & Diehl 2000). In addition, animal studies have shown that TNF-α initiates liver regeneration after partial hepatectomy by activating TNFR1 (Yamada et al. 1997). Despite the above observations that suggest a rather beneficial effect in the normal liver, TNF-α has been reported elevated in admission sera of patients with AH and its levels have been correlated with disease severity and mortality (Bird et al. 1990;Felver et al. 1990). Concurring animal studies have shown that TNFR1 knockout mice are resistant to alcohol induced liver damage (Yin et al. 1999) and that the administration of TNF-α antibodies prevents

liver injury in alcohol-fed rats (Iimuro et al. 1997). It is therefore believed that TNF-α biologic activities are altered in alcoholic liver disease, favouring inflammation and apoptosis rather than hepatocyte proliferation. The underlying molecular mechanisms are still unclear but ongoing research suggests that alterations in the NF-kB related signalling (Heyninck, Wullaert, & Beyaert 2003) probably influenced by the cytokine milieu and other pro-apoptotic pathways are involved (Stewart, Jones, & Day 2001;Tilg & Diehl 2000). The expression of the pro-inflammatory cytokine transforming growth factor-beta (TGF-β) is increased in alcoholic liver injury (Fang et al. 1998;Neuman et al. 2002) and has been shown to enhance hepatic fibrogenesis. TGF-β stimulates the activation of hepatic stellate cells which in turn promotes extra cellular matrix and collagen production within the hepatic parenchyma and leads the development of liver fibrosis. In addition, TGF-β has been shown to suppress the proliferation of hepatocytes as well as mediating hepatocyte apoptosis in alcohol induced liver injury (Gressner et al. 2002;Neuman 2003). Other chemokines such as IL-8, macrophage inflammatory protein 2 (MIP-2) (murine equivalent of human IL-8), cytokine induced neutrophil chemoattractant (CINC) (rodent equivalent of human IL-8) and MCP-1 and have all been shown to be elevated in alcohol induced liver injury (Fang et al. 1998; Sheron et al. 1993). Many different cells, including Kupffer cells and hepatocytes, produce these chemokines and it is thought that they participate in the recruitment of neutrophils to the site of alcohol liver injury by promoting neutrophil migration from the hepatic sinusoids through the endothelial lining and into the hepatic parenchyma (Purohit & Russo 2002). A potential mechanism by which this neutrophilic infiltration damages hepatic parenchymal cells is via neutrophil derived oxidant stress and protease enhanced cell injury. Adhesion molecules are also important factors in the migration of neutrophils and the primary adhesion molecules that participate in this process are selectin, β2 integrins, and intercellular adhesion molecule (ICAM)-1. The β2 integrins are expressed on neutrophils, whereas ICAM-1 is expressed on endothelial cells and hepatocytes (Purohit & Russo 2002).

The chemokine IP-10 is a potent chemoattractant for alloantigen-primed T cells (Farber 1997) and several studies have reported increased hepatic expression of this chemokine in animal models of alcoholic liver injury (Nanji et al. 1999). IP-10 is thought to play an important role in the activation of integrins on the cell surface of recruited lymphocytes, however the effects of IP-10 in alcohol induced liver injury remain obscure (Purohit & Russo 2002). Previous studies have demonstrated that acute alcohol consumption induces IL-10 production, a potent, immunoregulatory cytokine in human monocytes (Mandrekar et al. 2006;Szabo et al. 1996). One possible mechanisms by which ethanol use disturbs cellular immune responses may be a result of elevated IL-10 production by macrophages and T lymphocytes which promote humoral immune responses and inhibit cellular immune responses by down-regulating the production of Th1 cytokines, antigen-specific T cell proliferation, and proinflammatory cytokine levels including TNF-α (de Waal, Y et al.1993;Szabo et al. 2004). Although the immunomodulatory effects of IL-10 are widely investigated, the mechanisms of alcohol-induced IL-10 production are still not yet well defined.

## 1.7 Progenitor/stem cell response in alcohol induced liver injury

Rodent and human studies have demonstrated that alcohol toxicity provides a regenerative stimulus to the liver by; 1) inducing hepatocyte death, 2) inhibiting the proliferative activity of adult hepatocytes and 3) inducing the compensatory expansion of a liver progenitor cell/oval cell population (Diehl 2005;Natori et al. 2001;Ribeiro et al. 2004;Wands et al. 1979;Zhang, Gong, & Minuk 2000). Furthermore, both animal and human studies in chronic alcohol toxicity and liver injury have demonstrated that this facultative liver stem cell/progenitor cell population is activated in the presence and absence of inflammatory cell infiltrates and fibrogenesis and results in the regeneration of hepatocytes and biliary epithelial cells (Diehl 2005;Diehl, Tan 2002;Thorgeirsson, & Steer 1990;Roskams et al. 2003). These findings suggest that hepatic progenitor cell differentiation becomes a critical component of the regenerative response in alcohol

injured liver and that the requirement for differentiation is predicted to lengthen the time needed to accomplish regeneration. Current understanding on how chronic alcohol exposure modulates the viability and differentiation of hepatic progenitor cells is limited and virtually nothing is known about how alcohol affects the general mechanisms and processes that regulate these progenitor cells in adult livers (Diehl 2005).

It is unclear whether these progenitor/oval cell populations in adult livers are derived from an endogenous or a pluripotent circulating stem cell source (Menthena et al. 2004; Petersen et al. 1999;Wang et al. 2003a;Wulf et al. 2003). Hepatic progenitor cells/oval cells have some phenotypic traits that are typical of BM stem cells and the liver harbours many haematopoietic cell types (Omori et al. 1997;Petersen et al. 1998;Petersen et al. 1999). Rodent studies have demonstrated that BM derived hepatic oval/progenitor cells could differentiate into hepatocytes in 2-AAF / partial hepatectomy (PH) and 2-AAF/$CCl_4$ liver injury models which induce oval cell activation and proliferation (Oh et al. 2007; Petersen et al. 1999). Data is however lacking in both animal and human alcohol induced liver injury models on whether haematopoietic bone marrow stem cells can repopulate and transdifferentiate into hepatic progenitor/oval cells and give rise to hepatocytes.

Reports on the extent of peripheral blood HSC mobilisation in alcohol human induced liver injury is limited to two studies with very small patient numbers and only looking at patients with established alcohol cirrhosis and not acute alcoholic hepatitis. Kyriakou et al found no evidence of elevated peripheral blood HSCs levels in three alcohol cirrhotic patients whilst Di Campli et al failed to identify an increase in circulating HSCs in two patients with decompensated liver disease (Di Campli et al. 2005;Kyriakou et al. 2003). Studies on the extent of peripheral blood HSC mobilisation in acute alcohol induced liver injury have not been reported and the degree of contribution to liver repair from mobilised HSCs in this group of patients remains uncertain.

## 1.8 Bone marrow stem cell therapy in liver injury

For patients with end-stage liver disease or acute liver failure, whole organ liver transplantation is the only treatment of proven benefit. However the increase shortage of donor organs and the undesirable side effects of long-term immune suppressing drugs restrict its application to many patients and thus necessitates the development of alternative treatments. Whilst attention has been directed at developing alternative strategies such as bio-artificial liver systems and cellular transplantation (using hepatocyte suspensions), these still remain largely experimental. The application of BM stem cell therapy in liver disease is an attractive modality for the regeneration and reconstitution of damaged liver tissue, however it is still in its infancy with only a couple of un-controlled feasibility studies available. BM stem cells have been utilised to a greater degree in cardiology trials and demonstrating that therapy with adult BM derived cells reduces infarct size and improves left ventricular function and perfusion (Abdel-Latif et al. 2007; Erbs et al. 2007; Schachinger et al. 2006).

Intraportal administration of autologous $CD133^+$ bone marrow stem cells have been used to augment liver regeneration in patients with liver tumours who underwent portal vein embolisation to induce contralateral lobe hypertrophy prior to extensive partial hepatectomy (Am Esch et al. 2005). In this study, the computerised tomography (CT) scans demonstrated a 2.5 fold increase in size in the bone marrow-treated lobes when compared with the non-bone marrow cell-treated controls. To date only a handful of small phase 1 clinical trials have been reported in the literature involving autologous BM stem cell ($CD34^+$) infusions to patients with liver cirrhosis. Gordon et al conducted a preliminary uncontrolled study on 5 cirrhotic patients of varying aetiology and demonstrated an improvement in serum bilirubin and albumin levels 60 days after portal vein or hepatic artery infusions of autologous $CD34^+$ BM stem cells (Gordon et al. 2006). Two other published clinical trials demonstrated an improvement in cirrhotic patient clinical scores and biochemical parameters after autologous bone marrow cell

infusion therapy (Terai et al. 2006; Yannaki et al. 2006). The mechanism that underlies this improvement in not clear although it is speculated that HSCs may play a role in stimulating endogenous hepatocyte proliferation in a paracrine fashion (Yannaki et al. 2006). Large scale controlled trials are however lacking and further trials are needed to determine the effectiveness of stem cell infusion therapy in liver diseases.

## 1.9 Discussion

In this thesis alcohol induced liver injury was selected as the injury model of choice to study human HSC mobilisation and hepatic recruitment based on the following reasons. 1) Alcohol toxicity inhibits adult hepatocyte proliferation and promotes a regenerative stimulus to progenitor cells and potentially to BM derived pluripotent stem cells thus providing a suitable injury model in which to study circuilating HSC responses and hepatic recruitment. 2) Previous studies suggest that patients with inflammatory liver diseases have elevated circulating HSCs (autoimmune hepatitis) as well as BM stem cell contribution to liver repair (Hepatitis C). Acute alcoholic liver injury is another form of inflammatory liver disease worth studying as this injury model would provide further data to test the hypothesis that inflammatory liver injury promotes the release of stem cells from the BM and recruits them to the liver for repair. 3) Cytokines and inflammatory mediators such as IL-8, IP-10 and MCP-1 are thought to play a role in the pathogenesis of alcohol mediated liver injury, yet these very same cytokines are known to activate and induce the release of HSCs thus providing a possible mechanism to study and explain the regulatory process of BM stem cell release and recruitment in this form of liver injury. 4) Alcoholic liver disease is the commonest cause of cirrhosis in the Western world (Lieber 1993) and HSC multilineage plasticity has raised hopes that such cells could, in the future, be utilised for the regeneration and reconstitution of damaged liver tissue. This study will contribute to the understanding of the mechanisms involved in recruiting stem cells into injured liver and provide some clarification on how alcohol influences such processes, thus enabling us in the

future to manipulate these cells to develop new treatments for liver diseases including alcohol liver injury.

Current literature has demonstrated that ethanol exposure induces hepatocyte death and inhibits the proliferative response of adult hepatocytes resulting in the accumulation of hepatic stem cells within the liver as part of a regenerative response to injury although the precise origin of these cells (hepatic *vs.* BM-derived) remains unclear. Hepatic progenitor cells have some phenotypic traits that are typical of BM stem cells thus highlighting the limitation of performing immunohistochemical analysis alone for haematopoietic progenitor cells cell surface markers in alcohol injured liver tissue. Published data suggests that HSC contribution to liver repair varies considerably in various injury models however literature is lacking in both animal and human alcohol induced liver injury on whether haematopoietic bone marrow stem cells can actually be recruited into the injured liver and give rise to parenchymal cell lineages. Experimental Chapters 2 and 3 aims to establish whether alcohol induced liver injury in humans leads to the accumulation and expansion of progenitor stem cells within the liver and to demonstrate that they are from a bone marrow HSC source.

Recently it has been recognised that BM derived stem cells can also give rise to hepatic myofibroblasts, suggesting a functional contribution of BM stem cells to liver fibrosis (Forbes et al. 2004; Russo et al. 2006). However no data is yet available in alcohol induced liver injury whether BM derived stem cells contribute to either the hepatic parenchymal or hepatic myofibroblast population. Chapter 3 will attempt to address this unanswered question by assessing BM stem cell contribution to parenchymal and non parenchymal cell lineages in the alcohol injured liver and investigate the promotion of endogenous hepatocyte proliferation by haematopoietic stem cells.

Human studies have demonstrated elevated levels of peripheral blood HSCs in patients with extensive liver resections, autoimmune hepatitis and in liver transplant patients with ischaemic/reperfusion injuries. Data on the extent of peripheral blood HSC mobilisation in human alcohol induced liver injury is limited to a few individual patient cases with established alcohol cirrhosis but not to acute alcohol injury. Chapter 4 of this study would investigate and provide as yet unreported data on the extent of HSC mobilisation in patients with acute alcohol injury and attempt to provide some understanding on whether chemokine receptor expression of circulating HSCs are important in regulating this mobilisation process.

The cytokines SDF-1, MMP-9, G-CSF, IL-8, MIG, IP-10, RANTES and MCP-1 have been implicated in the mobilisation and migration of HSCs although their role in the mobilisation and recruitment of HSC in clinical liver injury including alcoholic liver disease has not yet been defined. In Chapter 5 we aim to study the role of these inflammatory cytokines and chemokine axes in the mobilisation and recruitment of HSCs in alcohol induced liver injury.

**Table 1**

| Tissue type | Cell Line SDF-1 Expression |
|---|---|
| Bone Marrow | Stromal Cell Lines |
| Tonsil | Epithelial Cells in Tonsillar Crypt |
| Spleen | Reticular Cells |
| Foetal Liver | Mesothelial Cells, Biliary Epithelium, Ductal Plate |
| Adult Liver | Biliary Epithelium |
| Lung | Interstitial Cells |
| Cardiac | Cardiac Myocytes |
| Brain | Glial Cells, Cortical Neuronal Cells, Astrocytes |
| Muscle | Skeletal Myocytes |
| Skin | Epithelial Cells of Sweat Glands, Endothelial Cells, Pericytes, Dendritic Cells |
| Thymus | Stromal Cells, Medullary Cells, Epithelial Cells |

**Table 1: SDF-1 expression in normal human tissue.**

**Figure 1A-C**

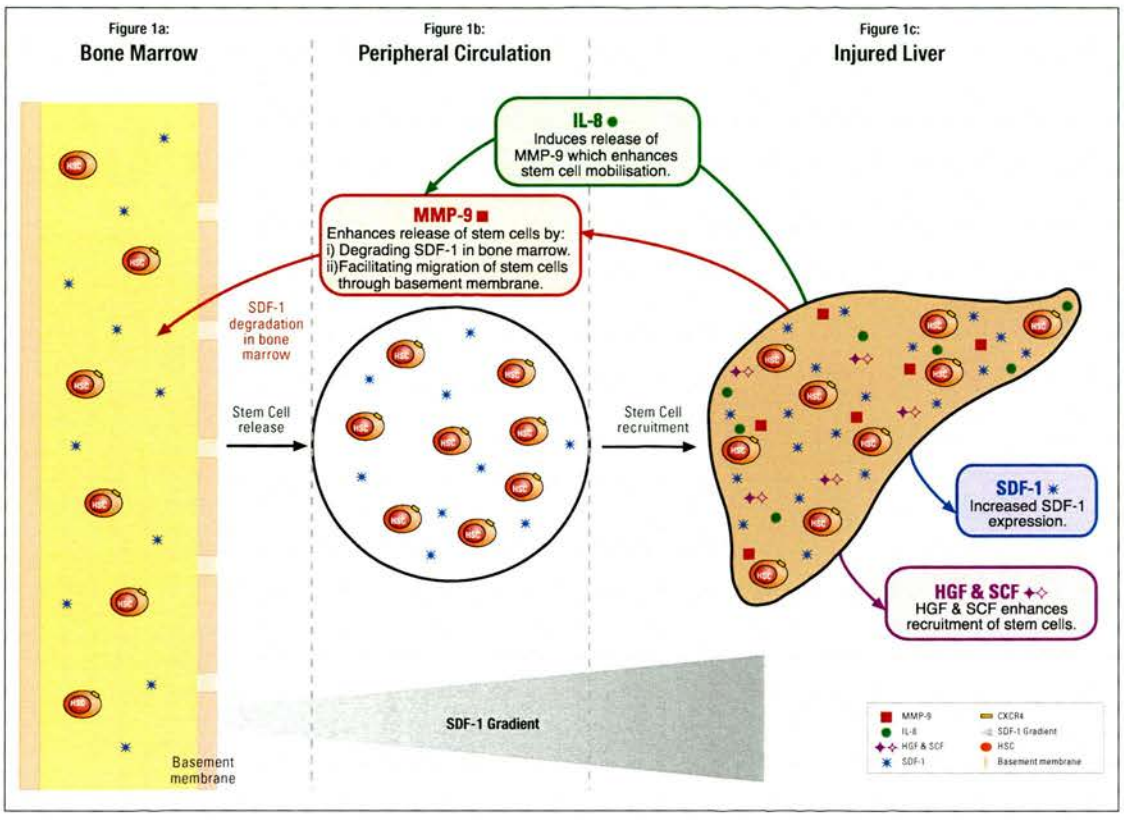


**Figure 1A:** Release of HSCs from bone marrow into peripheral circulation mediated via SDF-1 concentration gradient.

**Figure 1B:** Release of HSCs into peripheral circulation enhanced via MMP-9 and IL-8.

**Figure 1C:** Recruitment of HSCs into injured liver mediated via SDF-1, HGF and SCF.

# CHAPTER 2

## Hepatic stem cell content in human alcohol induced liver injury

## 2.1 Introduction

The significance of the contribution HSCs make to liver repair is contentious and studies in both rodents and humans have demonstrated that ethanol exposure induces hepatocyte death and results in the accumulation of hepatic progenitors/oval cells within the liver as part of a regenerative response to injury (Diehl 2005; Diehl, Thorgeirsson & Steer 1990; Roskams et al. 2003). The precise origin of these progenitor cells (hepatic *vs.* BM-derived) however remains controversial (Menthena et al. 2004; Petersen et al. 1999; Wang et al. 2003a; Wulf et al. 2003). The aim of this chapter is to establish whether alcohol induced liver injury leads to the accumulation and expansion of progenitor stem cells within the liver which express haematopoietic stem cell markers ($CD34^+$ and c-$kit^+$).

## 2.2 Patients and methods

### 2.2.1 Patient details

In this study we analysed archival liver biopsy tissue specimens from 5 adult normal control (NC) samples and compared them with 4 patients who had a history of continuous alcohol consumption > 400gms/week, no clinical or serological evidence of other causes of liver disease and had histological evidence of alcoholic hepatitis (AH) on biopsy. The following histological criteria were used for the diagnosis of AH in this study; 1) Macrovesicular and microvesicular steatosis, 2) polymorphonuclear cellular infiltrate, 3) intracellular accumulation of Mallory's hyaline/bodies and 4) evidence of hepatocellular injury or necrosis.

Ethical approval for the use of archival liver biopsy tissue in this study was granted by the Lothian Health Board Research and Ethics Committee (2003/R/GI/02). Sample sizes for the control and AH patient groups was limited in this study due to the restrictions placed by the ethical approval which only authorised access to a set number of 75 pre-determined archival liver biopsy tissue samples of various hepatic aetiologies. This meant that only 4 samples that fulfilled the criteria for alcohol related hepatitis and 5 NC samples could be identified from this set. The anonymisation process of the archival liver biopsies resulted in us being unable to retrieve further patient/clinical details for 3 out of the 5 control specimens labelled as normal. These NC samples labelled 1-3 were re-examined by a pathologist (Prof DJ Harrison) and verified histologically that they appeared normal although we appreciate the limitations of not having more clinical details to substantiate this clinically. The NC samples 4 and 5 were from pre transplant female donor livers aged 47 and 62 years respectively, both died of subarachnoid haemorrhage and had no clinical history of pre-existing liver disease. Both tissue samples were documented as normal on histopathology reporting. The 4 AH liver biopsies specimens were from 1 female aged 52 and 3 male patients

aged 38, 48, and 31 years respectively. Histopathological diagnosis of all liver samples were based on the original pathological report and re-examined by a pathologist (Prof DJ Harrison).

### 2.2.2 Immunohistochemistry for hepatic stem cells

To identify hepatic progenitor stem cells within the liver which express haematopoietic stem cell markers ($CD34^+$ and c-$kit^+$), immunohistochemical staining was performed sequentially on formalin fixed paraffin embedded (FFPE) tissue sections using selected antibodies. A total of 4 consecutive sections were obtained from each biopsy specimen. Each section was 3 micron thick, which is approximately half the thickness of a nucleus, with neighbouring sections cut 3 microns apart. This procedure allowed matching fields to be as close as possible to each other. Sections were dewaxed in xylene for 10 minutes and rehydrated through graded alcohols (100%, 90% and 70%) for 5 minutes each. All tissue sections were washed in distilled water for 5 minutes and then microwaved in a pressure cooker for 4 minutes in 0.372gm/L EDTA (pH8) solution. After microwave antigen retrieval the slides were allowed to cool in water for 5 minutes and then blocked in 1% hydrogen peroxide for 15 minutes. Slides were loaded into sequenza trays and washed with phosphate buffered saline (PBS). Each slide was incubated for 30 minutes at room temperature with a single selected primary antibody: CD31 mouse mAb (DakoCytomation, N1596), CD34 mouse mAb (DakoCytomation, N1632), c-kit rabbit mAb (DakoCytomation, A4502) and mast cell tryptase mouse mAb (DakoCytomation, M7052) at a dilution of 1:50, 1:50, 1:100 and 1:1600 respectively ***(see figure 2)***.

The CD34, CD31 and mast cell tryptase labelled slides were washed and incubated for 30 minutes at room temperature with a horseradish peroxidase (HRP) polymer-conjugated detection antibody (DakoCytomation EnVision™ + System-HRP, K4006/ K4010). The c-kit labelled slides were washed and incubated for 30 minutes at room

temperature in a 1:400 dilution of biotinylated swine anti-rabbit immunoglobulins (DakoCytomation, E0353) followed by wash of PBS and a further 30 minute incubation at room temperature with an Avidin-Biotin HRP reagent (Vectastain Elite ABC kit, PK6100). All slides were washed and incubated for 5 minutes at room temperature with either diaminobenzidine (DAB) (DakoCytomation) or Vector® Nova RED™ as substrate chromagens and then counterstained with Mayer's haematoxylin.

### 2.2.3 Microscopy and image processing

Each tissue section was stained with only a single antibody and sections were viewed under a light microscope (Olympus B061, Japan). Sections were screened on low power (200x) and areas of positive cell staining within portal tracts were photographed at a magnification of 200x and 400x using a colour digital camera (Pixera Penguin Pro 150ES, USA). Serial composite images of each photographed section were pseudocoloured *(green)* and processed using image processing software (Adobe® Photoshop® Software). CD34 is not exclusively expressed on haematopoietic stem cells as vascular endothelial cells also express CD34 (Fina et al. 1990). Likewise c-kit is expressed on haematopoietic progenitors and mast cells (Baghestanian et al. 1996). With Adobe® Photoshop® we were able to distinguish the true progenitor stem cells from the endothelial ($CD31^+$) and mast cells (mast cell tryptase) by digitally subtracting the $CD31^+$ and mast $cell^+$ images from the composite images.

### 2.2.4 Cell counting

True progenitor stem cells were determined by counting $c\text{-}kit^+$/$mast\ cell^-$ & $CD34^+$/ $CD31^-$ cells from the processed digital images. Two separate assessors counted all sections from the digital images taken at 400x magnification and were blinded to the nature of the biopsy. The sections being measured contained a minimum of 3 portal tracts each and the true progenitor cells were quantified as the number of cells per triad per patient.

### 2.2.5 Statistics

All statistics were performed on SPSS® 12.0 for Windows® software (SPSS Inc. Chicago, IL). Results are expressed as the Mean with Standard Error of the Mean (SEM) for individual column data. The Mann-Whitney U statistical test (non-parametric significance) was used to determine whether the differences between the NC and AH progenitor stem cell counts were significant. Results were considered significant when $p<0.05$. The Cohen's kappa coefficient was used to determine inter-observer agreement on true progenitor stem cell counts in the liver sections.

## 2.3 Results

### 2.3.1 Increased numbers of stem cells ($CD34^+/CD31^-$ and c-$kit^+$/mast $cell^-$) are seen in the livers of patients with alcoholic hepatitis

Immunostaining demonstrated that liver biopsies from AH patients contained a greater number of stem cells/hepatic progenitor cells ($CD34^+/CD31^-$ & c-$kit^+$/mast $cell^-$) in the periductal region of the portal tracts as compared with NC. The $CD34^+/CD31^-$ population was significantly elevated in the AH group (1.5 cells/triad/patient ± 0.58, $p<0.05$) as compared to the NC group (0.24 cells/triad/patient ± 0.14). Likewise, the c-$kit^+$/mast $cell^-$ population of hepatic progenitor/stem cells was significantly elevated in patients with AH (1.3 ± 0.33 cells/triad, $p<0.05$) as compared to the NC group (0.52 ± 0.18 cells/triad) ***(see figures 3-7)***. The ratio of $CD34^+$ to c-$kit^+$ stem cells in the AH patient group was 0.87:1 and in the NC group 0.46:1. The Cohen's kappa coefficient for the inter-observer agreement on true progenitor stem cell counts was 0.79.

## 2.4 Conclusion

We have demonstrated that in alcohol induced liver injury there is an expansion of hepatic progenitor cells within the liver expressing HSC markers and suggesting a possible bone marrow source. We do however acknowledge the limitations of this study in particular to the small sample size and the lack of clinical data available on some of the normal control samples. Furthermore, hepatic progenitor cells express a large panel of cell surface markers and share cell surface markers such as Thy-1(CD90), c-kit, and CD34 with haematopoietic progenitor cells (Omori et al. 1997; Petersen et al. 1998; Petersen et al. 1999) thus highlighting the limitation of performing immunohistochemical analysis alone to distinguish the origins of these progenitor cells (Bone marrow *vs.* endogenous). In Chapter 3 we aim to establish that circulating bone marrow derived stem cells are recruited into the alcohol injured liver by performing immunohistochemistry and fluorescent *in situ* hybridisation (FISH) for recipient derived Y-chromosome on liver biopsies samples from cross-sex liver transplant patients who subsequently developed evidence of alcohol induced liver injury.

**Figure 2**

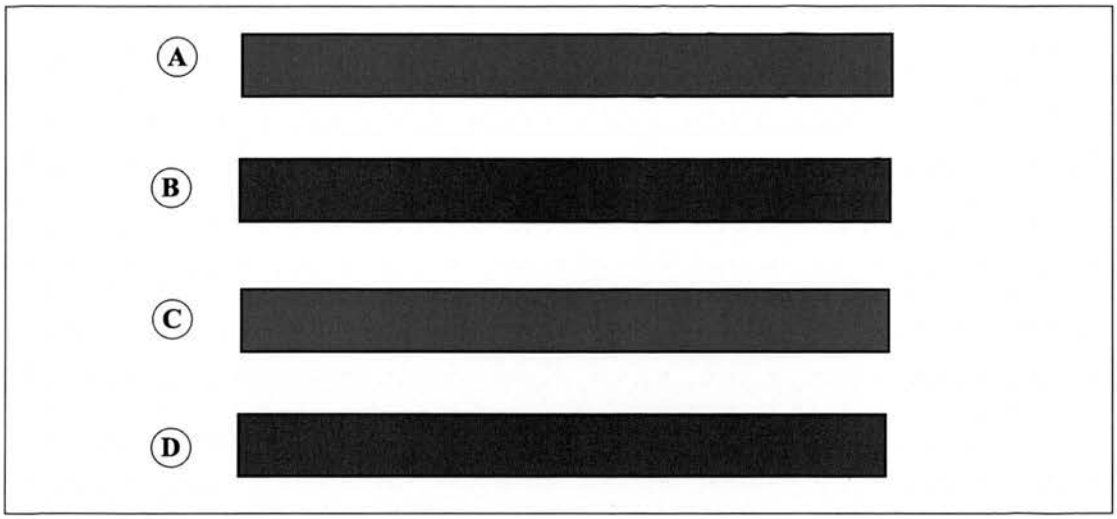


**Figure 2: Immunohistochemical analysis for stem cells in liver sections.**

Serial 3-µm-thick sections stained in the following sequence with primary antibodies. **A)** CD31-staining for endothelial cells. **B)** CD34-staining for HSCs and endothelial cells. **C)** Mast cell tryptase-staining for mast cells. **D)** C-kit-staining for HSCs and mast cells.

**Figure 3**

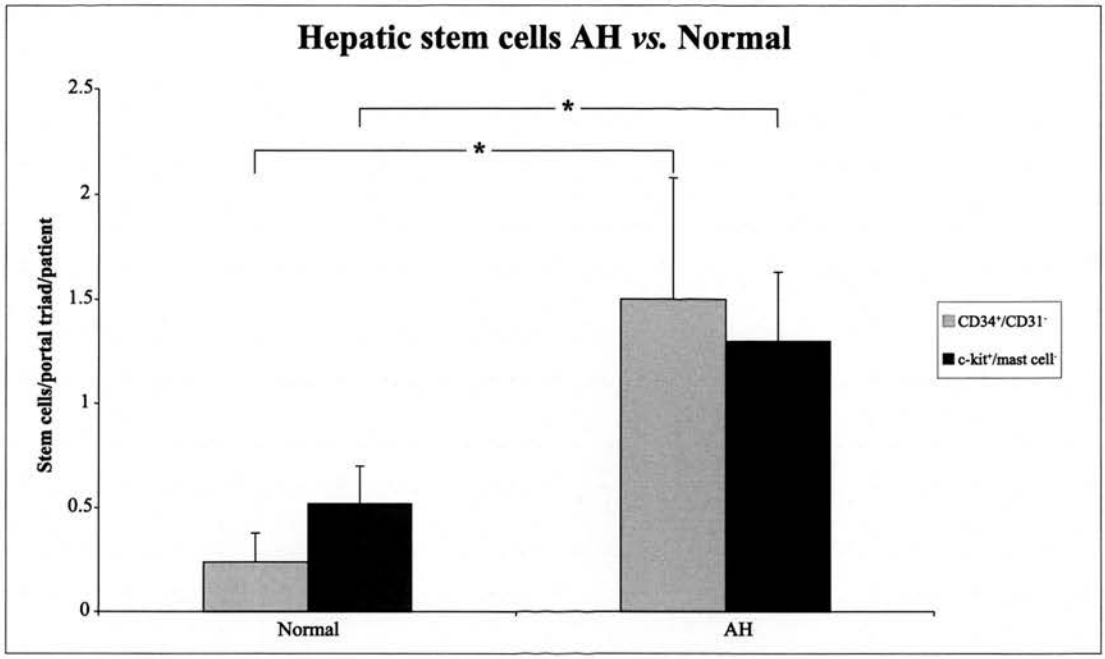


**Figure 3: Hepatic stem cell ($CD34^+/CD31^-$ and $c\text{-}kit^+/mast\ cell^-$) populations are elevated in AH patients.**
Values are expressed as stem cells per portal triad per patient. Data represents mean values for each group ± SEM. *$p<0.05$ compared with normal value.

**Figure 4**

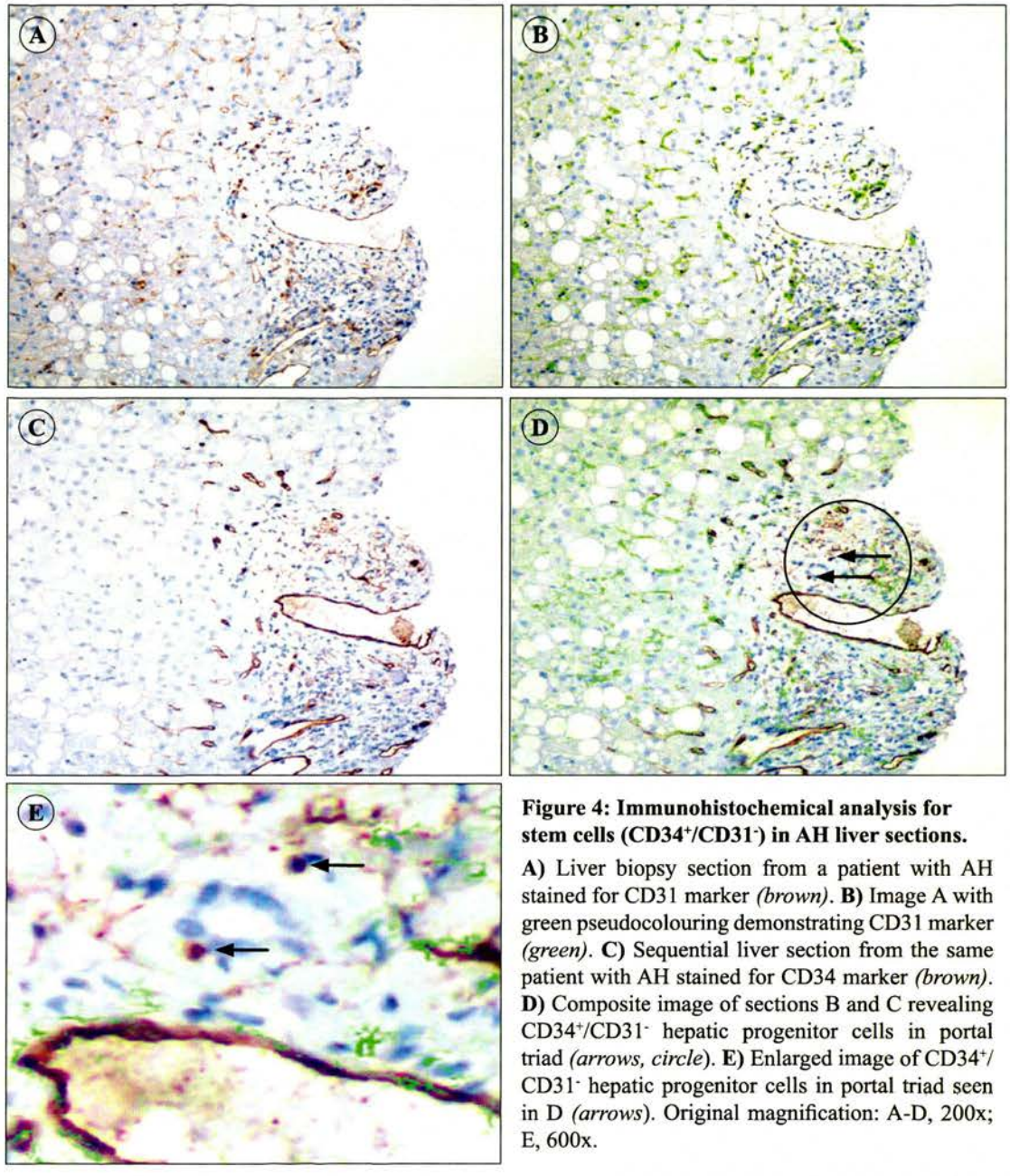


**Figure 4: Immunohistochemical analysis for stem cells ($CD34^+/CD31^-$) in AH liver sections.**

**A)** Liver biopsy section from a patient with AH stained for CD31 marker *(brown)*. **B)** Image A with green pseudocolouring demonstrating CD31 marker *(green)*. **C)** Sequential liver section from the same patient with AH stained for CD34 marker *(brown)*. **D)** Composite image of sections B and C revealing $CD34^+/CD31^-$ hepatic progenitor cells in portal triad *(arrows, circle)*. **E)** Enlarged image of $CD34^+/CD31^-$ hepatic progenitor cells in portal triad seen in D *(arrows)*. Original magnification: A-D, 200x; E, 600x.

**Figure 5**

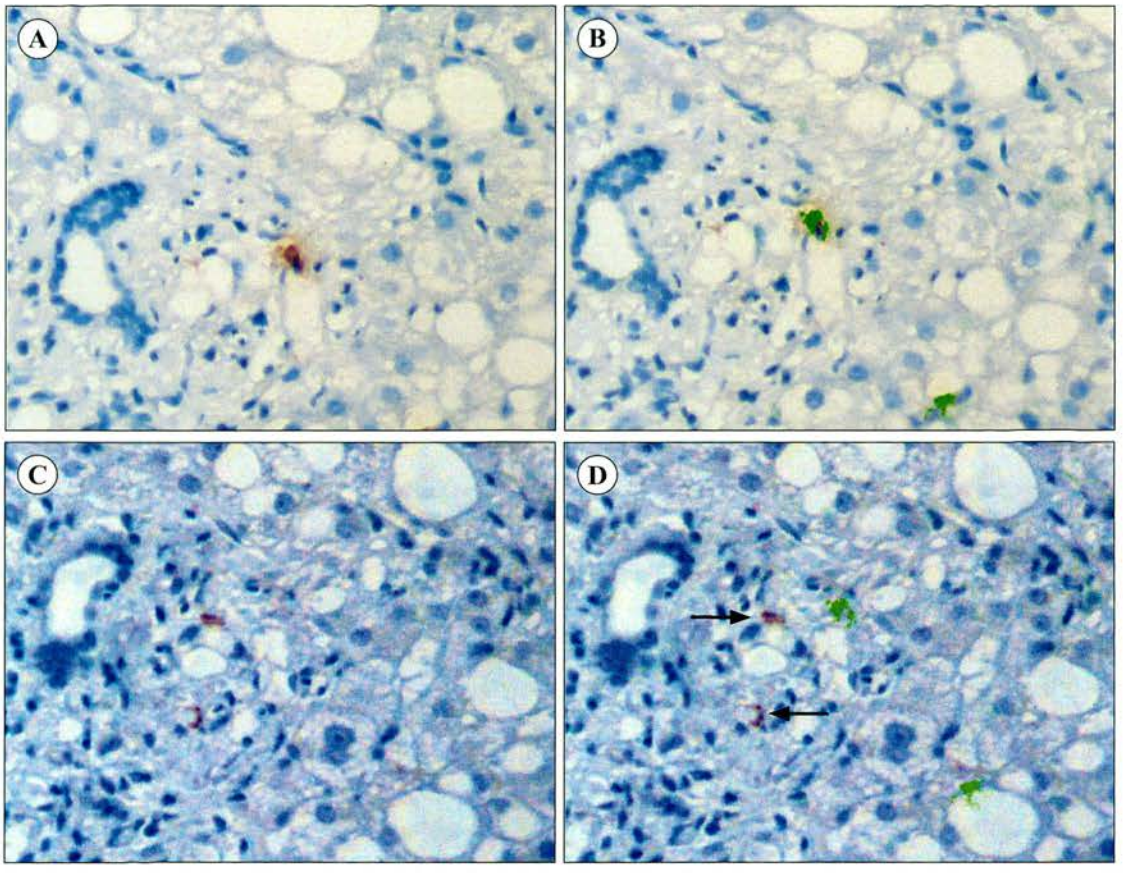


**Figure 5: Immunohistochemical analysis for stem cells (c-kit$^+$/mast cell$^-$) in AH liver sections.**

**A)** Liver biopsy section from a patient with AH stained for mast cells *(brown)*. **B)** Image A with green pseudocolouring demonstrating mast cells *(green)*. **C)** Sequential liver section from the same patient with AH stained for c-kit marker *(brown)*. **D)** Composite image of sections B and C revealing c-kit$^+$/mast cell$^-$ hepatic progenitor cells in portal triad *(arrows)*. Original magnification: A-D, 400x. These images have been magnified to demonstrate stem cell morphology.

**Figure 6**

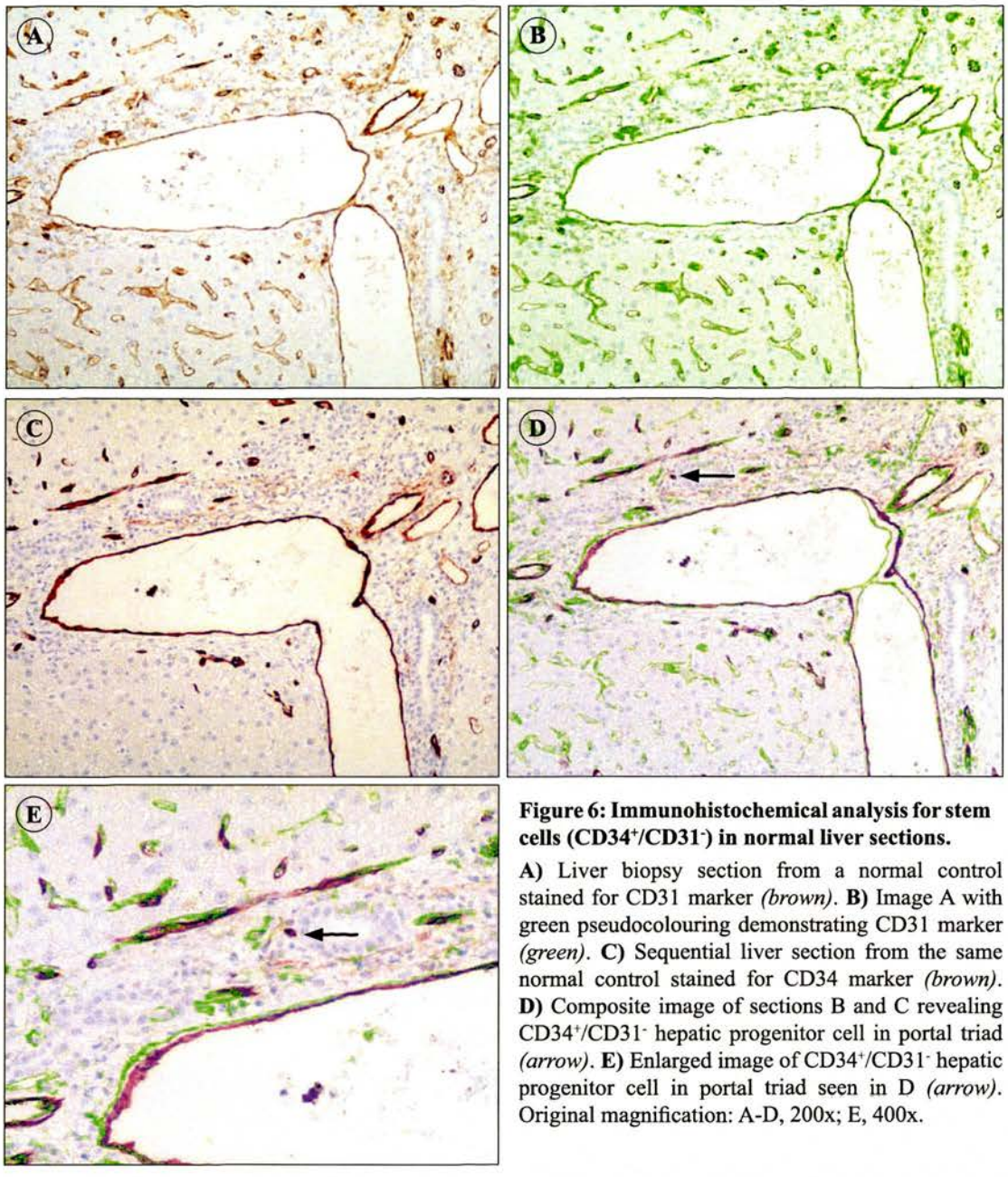


**Figure 6: Immunohistochemical analysis for stem cells ($CD34^+/CD31^-$) in normal liver sections.**

**A)** Liver biopsy section from a normal control stained for CD31 marker *(brown)*. **B)** Image A with green pseudocolouring demonstrating CD31 marker *(green)*. **C)** Sequential liver section from the same normal control stained for CD34 marker *(brown)*. **D)** Composite image of sections B and C revealing $CD34^+/CD31^-$ hepatic progenitor cell in portal triad *(arrow)*. **E)** Enlarged image of $CD34^+/CD31^-$ hepatic progenitor cell in portal triad seen in D *(arrow)*. Original magnification: A-D, 200x; E, 400x.

**Figure 7**

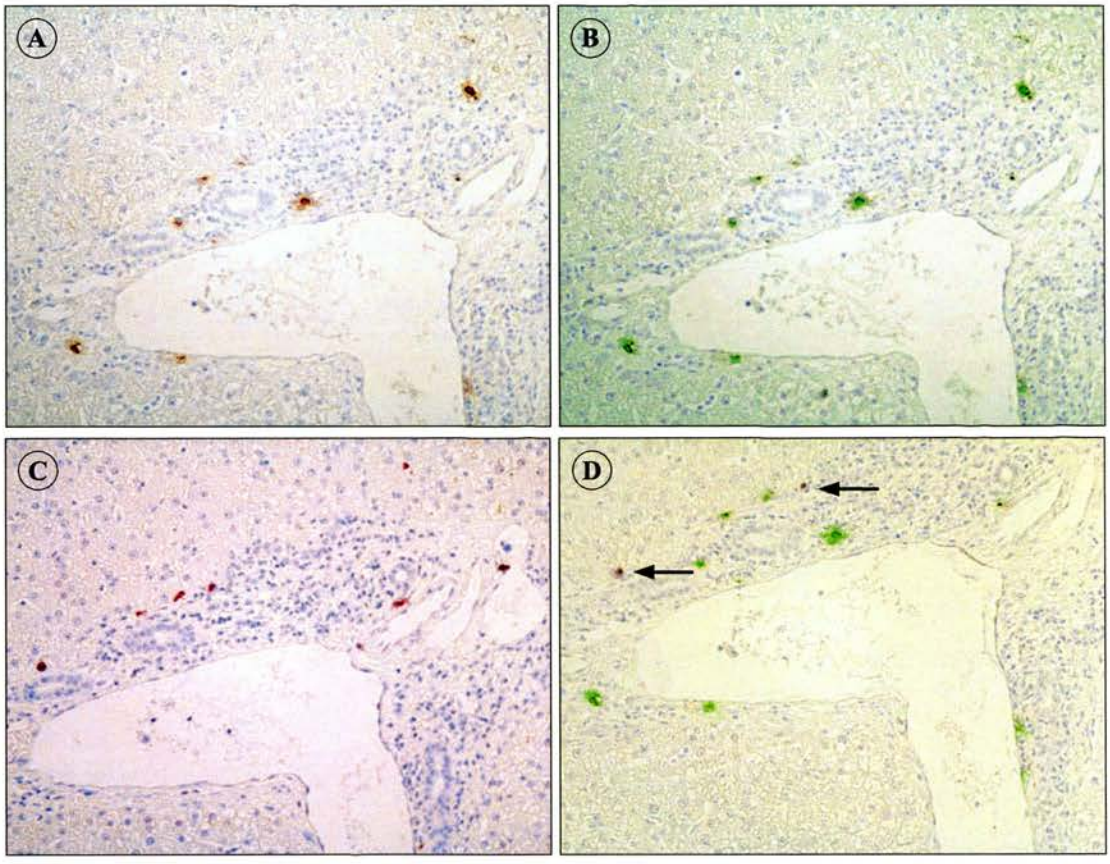


**Figure 7: Immunohistochemical analysis for stem cells (c-kit$^+$/mast cell$^-$) in normal liver sections.**

**A)** Liver biopsy section from a normal control stained for mast cells *(brown)*. **B)** Image A with green pseudocolouring demonstrating mast cells *(green)*. **C)** Sequential liver section from the same normal control stained for c-kit marker *(red)*. **D)** Composite image of sections B and C revealing c-kit$^+$/mast cell$^-$ hepatic progenitor cells in portal triad *(arrows)*. Original magnification: A-D, 200x.

# CHAPTER 3

## Hepatic recruitment of bone marrow derived stem cells in human alcohol induced liver injury

## 3.1 Introduction

HSC contribution to liver repair varies considerably, with some of the observed variation and putative mechanisms (transdifferentiation and fusion) being possibly related to the type and severity of liver injury (Lagasse et al. 2000; Sakaida et al. 2004b; Terai et al. 2003). HSCs may also play a role in stimulating endogenous hepatocyte proliferation in a paracrine fashion as evidenced by their contribution in rodent (Yannaki et al. 2005) and human studies (Terai et al. 2006). Recently it has been recognised that BM derived stem cells can also give rise to hepatic myofibroblasts, suggesting a functional contribution of BM stem cells to liver fibrosis (Forbes et al. 2004; Russo et al. 2006). Data is however lacking in both animal and human alcohol induced liver injury models on whether haematopoietic bone marrow stem cells can repopulate the liver and transdifferentiate into hepatocytes and/or contribute to the myofibroblast population.

In the previous chapter **(Chapter 2)** we demonstrated that in alcohol induced liver injury there is an expansion of hepatic progenitor cells expressing HSC markers and suggesting a possible bone marrow source. In this chapter we wanted to establish that alcohol induced liver injury, increases the hepatic recruitment of bone marrow derived stem cells into the injured liver as well as to assess their contribution to parenchymal and non parenchymal cell lineages in the liver.

## 3.2 Patients and methods

### 3.2.1 Patient details

In this study, cross-sex liver transplant patients who subsequently developed evidence of alcohol induced liver injury were used to study the contribution of bone marrow derived HSCs to parenchymal and non parenchymal liver cell lineages. Ethical approval for the use of archival liver biopsy tissue in this study was granted by the Lothian Health Board Research and Ethics Committee (LREC/2002/8/25). and informed consent was obtained from patients to use the liver biopsy samples for experimental purposes. We analysed archival liver transplant biopsies tissue samples taken at the Royal Infirmary of Edinburgh from the period 1992 to 2003 from male patients who were transplanted with female donor livers. From 90 archival cross-sex transplant liver biopsy samples we could only identify 3 male cross-sex liver transplant patients who subsequently developed evidence of alcohol induced liver injury ranging from steatosis to steatohepatitis.

Patient characteristics, time of biopsy post transplant and histological diagnosis are summarised in ***table 2***. Histologically all 3 specimens showed features of alcohol induced liver injury with no evidence of acute or chronic rejection. Patient 1 was a 59 year old male, non diabetic, BMI 27.8, with a history of alcohol consumption post transplant and histology demonstrating a mild lobulitis with marked microvesicular steatosis. No spotty necrosis or Mallory's hyaline was identified. No other cause for fatty liver disease was identified. Patient 2 was a 50 year old male with a history of alcoholic liver disease and chronic hepatitis C and was treated with 6 months treatment of Interferon/Ribavirin therapy 36 months prior to liver transplantation. At the time of liver transplantation his hepatitis C PCR was negative and remained negative at the time of repeat liver biopsy. Unfortunately HCV Genotyping was not available in the clinical notes, laboratory database or HCV patient treatment database. Histology

demonstrated microvesicular steatosis, mild cholestasis related changes and giant mitochondria favouring alcohol induced liver injury. Patient 2 was non diabetic, BMI 20.9 and no other cause for fatty liver disease was identified. Patient 3 was a 46 years old male, non diabetic, BMI 24.8 with a history of alcohol binge drinking post transplant. Histology demonstrated severe mixed microvesicular and macrovesicular steatosis, low grade peri-portal lymphocytic infiltrate, degeneration of centrilobular hepatocytes and Mallory's hyaline consistent with alcoholic hepatitis. Routine day 7 post cross-sex liver transplant biopsies of these 3 patients were included as controls as well as 3 normal pre transplant donor male liver controls and 3 normal pre transplant donor female liver controls. The day 7 post transplant biopsies did not demonstrate any significant cellular rejection or reperfusion injury. Histopathological diagnosis of all liver samples were based on the original pathological report and re-examined by a pathologist (Prof DJ Harrison).

### 3.2.2 Combined Immunohistochemistry and Fluorescent *in situ* Hybridisation (FISH) for Y-chromosome

All liver sections were FFPE and cut sequentially 3 microns apart. Each section was 3 micron thick, which is approximately half the thickness of a nucleus, thus allowing for matching fields to be as close as possible to each other. Slides were heated to 60°C for 20 minutes to melt wax, then washed in xylene 4 times for 10 minutes each before dehydration through an ethanol series (100%, 95% and 70%). Slides were then microwaved for 20 minutes in 0.1 mol/L citrate buffer, pH6.0 washed with PBS and blocked in 1% hydrogen peroxide for 15 minutes. Slides were incubated for 30 minutes at room temperature with one of the following antibodies; 1) HSC marker: CD34 mouse anti human antibody 1:10 dilution (DakoCytomation, N1632), 2) biliary epithelial marker: AE1/AE3 mouse antihuman antibody 1:50 dilution (DakoCytomation, M3515), and 3) myofibroblast marker: α-smooth muscle actin (α-SMA) mouse antihuman antibody 1:50 dilution (DakoCytomation, M0851). Slides were also incubated overnight at

4°C with Ki-67 mouse antihuman antibody 1:400 dilution (DakoCytomation, M7240) which is a marker for increased hepatocyte regeneration (Crary & Albrecht 1998). After primary antibody incubation slides were washed and incubated for 30 minutes with an HRP polymer-conjugated detection antibody (DakoCytomation EnVision™ + System-HRP, K4006/K4010) or an AP detection antibody (DakoCytomation, K1396) followed by incubation with either DAB (DakoCytomation) or Vector® Nova RED™(Vector Labs, SK5100) as substrate chromagens. CD34 and α-SMA stained slides were co-labelled with: CD31 mouse antihuman mAb (DakoCytomation, N1596) and CD34 mouse antihuman mAb (DakoCytomation, N1632) at a 1:10 dilution followed by AP detection antibody (DakoCytomation, K1396) and Vector® Nova RED™ (Vector Labs, SK5100).

One microgram human Y-chromosome DNA was labelled with digoxigenin 11-dUTP (Roche, 1363905), by nick translation. Unincorporated nucleotides were removed by centrifugation through Quick Spin G50 Sephadex columns (Roche, 1273973). Specific activity of the probes was performed by dotting onto nitrocellulose filters using anti-digoxigenin-alkaline phosphatase Fab fragments (Roche, 1093274). We then used a BCIP/NBT kit (Vector, SK 5400), which produces the purple change on the filter, which we then compared with known standards (DNA of known concentrations). After thorough washing the liver sections were denatured for 3 minutes at 75°C in 70% formamide/2x SSC, plunged into ice-cold 70% ethanol for 3 minutes, dehydrated through an alcohol series and air-dried. Two hundred nanograms labelled human Y-chromosome DNA, 10μg Cot1 DNA (repetitive DNA sequences) and 5μg sonicated salmon sperm DNA were denatured together for 5 minutes at 70°C in 50% deionised formamide, 50% dextran sulphate, 2x SSC and 1% Tween 20. Thereafter the hybridisation mix and probe were re-annealed at 37°C for 15 minutes. 10μl of mix (hybridisation mix and probe) was pipetted onto each slide and allowed to hybridise overnight at 37°C. Slides were washed 4 times for 3 minutes in 2x SSC at 45°C and

then successively incubated for 30 minutes at 37°C with a 1:500 dilution of a sheep avidin Fluorescein Isothiocynate (FITC) antibody (Roche), followed by a further 30 minutes with a 1:100 dilution of anti avidin biotin antibody and then a final 30 minutes with a 1:500 dilution of a sheep avidin FITC antibody (Roche). The slides were washed thoroughly in 4x SSC/0.1% Tween 20 between incubations. Slides were mounted in Vectashield (Vector Laboratories) containing 1g/mL 4, 6-diaminidino-2-phenylindole (DAPI) counterstain.

Hepatocyte immunostaining quality was compromised by the *in situ* hybridisation processing methods which affected the hepatocyte epitopes in these liver sections. hybridization process, thus we employed a method of processing the 3 micron thick serial sections sequentially for Y-chromosome FISH followed by immunohistochemistry for Hep Par 1 hepatocyte mouse antihuman mAb 1:50 dilution (DakoCytomation, M7158) as per above protocol.

### 3.2.3 Microscopy and image capture

For light microscopy, an Olympus B061 microscope was used and images captured with a digital camera (Pixera Penguin Pro 150ES, USA).For fluorescent microscopy, slides were visualised using a Zeiss Axioplan 2 fluorescence microscope equipped with a triple bandpass filter (Chroma #83000). Greyscale images were collected with a cooled charge-coupled devise camera (Quantix Corp) and analysed using custom IPLab scripts. Image processing was performed using Adobe® Photoshop® software.

### 3.2.4 Cell counting

Y-chromosome positive nuclei were counted from contiguous fields at x 400 magnification and matched to the corresponding immunostaining for CD34 / CD31 / AE1/AE3 / α-SMA / and Hep Par 1 markers according to the location and architecture of the tissue on the slide. Counting of all Y-chromosome positive nuclei in a 3 micron

thick section was not possible because a significant proportion of Y-chromosome will be missed by the section in any particular cell. Thus a correction factor was obtained by counting all nuclei from normal male liver control tissue and expressed as a fraction of $Y^+$/total number of nuclei. Analysis of the normal male liver control group identified only 38.46% of all nuclei as being Y-chromosome positive and a correction factor of 2.60 was applied to all $Y^+$ cell counts in order to obtain a true estimate of male recipient derived cells in each section. The above methodology has commonly been applied in other similar studies (Theise et al. 2000b;Forbes et al. 2004). Both corrected and absolute $Y^+$ cell counts were analysed for statistical purposes in order to ensure that any errors that may have occurred in the assessment of Y-chromosome detection was not exaggerated and multiplied.

To evaluate the percentage of positive staining Ki-67 hepatocytes, 5 to 10 random fields were selected per liver biopsy slide and Ki-$67^+$ hepatocytes were counted under x400 magnification. A single assessor counted all sections from the digital images and was blinded to the nature of the biopsy.

### 3.2.5 Statistics

All statistics were performed on SPSS® 12.0 for Windows® software (SPSS Inc. Chicago, IL). Results are expressed as the Mean with Standard Error of the Mean (SEM) for individual column data. The Wilcoxon Matched Pairs Signed Ranks test (non-parametric paired testing significance) was used to determine whether the differences between the day 7 post transplant biopsies with the subsequent alcohol liver injury biopsies were significant. Results were considered significant when $p<0.05$.

## 3.3 Results

### 3.3.1 A significant proportion of $CD34^+$ and $\alpha$-$SMA^+$ cells in alcohol injured livers are derived from circulating bone marrow cells

To identify the source of the intrahepatic $CD34^+$ stem cells in the context of alcohol induced liver injury, we studied cross sex liver transplant biopsies from male patients with evidence of alcohol induced liver injury using combined FISH for Y-chromosome and immunohistochemistry for $CD34^+/CD31^-$ cells. Analysis of the normal male liver control group identified Y-chromosome positive nuclei whilst no Y-chromosome positive nuclei were detected in any of the female donor livers prior to transplantation (3/3 normal histology) ***(see figure 8)***.

There were increased $Y^+$ recipient $CD34^+$ cells (expressed as a % of total cells) in the cross-sex liver grafts from patients with alcohol induced liver injury than in control patients who had a routine day 7 post transplant protocol liver biopsy ($0.706 \pm 0.233\%$ *vs.* $0.036 \pm 0.02065\%$ $p<0.05$).When this figure was corrected by the correction factor of 2.60 (see methodology) then the $Y^+$ recipient $CD34^+$ cell count in the alcohol injured cross-sex liver grafts compared with routine day 7 post transplant protocol liver biopsy was $1.834 \pm 0.605\%$ *vs.* $0.094 \pm 0.054\%$, $p<0.05$. These $Y^+/CD34^+$ cells were identified within the perisinusoidal, intrahepatic and periportal regions of the biopsies whilst none were seen within the biliary tracts ***(see figures 9-10).*** These $Y^+/CD34^+$ cells did not co-stain for the endothelial cell marker CD31 suggesting they were true BM derived stem cells and not donor derived endothelial cells ***(see figure 11).*** Further analysis in the 3 cross-sex liver grafts samples with alcohol induced liver injury demonstrated that 7.79%, 3.03% and 10.3% of the myofibroblasts ($\alpha$-$SMA^+$) were $Y^+$. When this figure was corrected by the correction factor of 2.60 then 20%, 7.9% and 26.8% of the myofibroblasts seen were of BM origin ***(see figures 12-13)***, with the value of 26.8% being found in the Patient 3 sample with the most pronounced histological changes.

Notably the proportion of α-SMA$^+$ cells that co-stained for CD34 was higher in cross-sex donor liver grafts with alcohol induced as compared with controls (5.025 ± 0.621% *vs.* 0.494 ± 0.197%, $p<0.05$) ***(see figures 14-15)***. We were unable to identify any Y$^+$ biliary epithelial cells or hepatocytes in these biopsies suggesting that BM derived stem cells did not contribute to hepatocyte or biliary cell repair in alcohol induced liver injury ***(see figure 16)***.

### 3.3.2 Increased stem cell numbers in alcohol injured livers are not associated with an alteration in endogenous hepatocyte proliferation.

Liver regeneration in the cross-sex liver biopsy samples was assessed by immunohistochemical analysis of Ki-67 staining. We did not detect any increase in the percentage of Ki-67$^+$ staining hepatocytes in the cross-sex liver grafts with AH as compared with controls (2.088 ± 0.410% *vs.* 1.884 ± 0.285%), suggesting that HSCs recruited into the liver do not promote endogenous hepatocyte proliferation in a paracrine fashion ***(see figures 17-18)***. Results for combined FISH and immunostaining analysis are summarised in ***table 3***.

## 3.4 Conclusion

From this study we have demonstrated that human BM derived stem cells are recruited into the liver during alcohol induced liver injury and that a proportion of myofibroblasts post liver transplantation are derived from the BM in whom alcohol injury has recurred, consistent with previous observations post transplantation in viral hepatitis. Furthermore, it is also possible that a population of $CD34^+$ cells within the liver were contributing to the recipient derived myofibroblast population in alcohol liver injury. Whilst there was an increase in the $CD34^+$ stem cell population within the liver there was no evidence of increased hepatocyte proliferation nor evidence of BM stem cell contribution to parenchymal cell lineages in the alcohol injured liver. We appreciate that the number of patients studied to assess the contribution of BM stem cells to hepatic myofibroblasts and progenitor populations is small, although recurrence of alcohol induced liver injury is an uncommon clinical feature in post transplant patients thus limiting the availability of such samples to study.

**Table 2**

| | **Patient 1** | **Patient 2** | **Patient 3** |
|---|---|---|---|
| Age of recipient (years) | 59 | 50 | 46 |
| Aetiology for transplantation | ALD | HCV & ALD | ALD |
| Blood group of recipient | $O^+$ | $A^+$ | $A^+$ |
| Sex of donor | Female | Female | Female |
| Age of donor (years) | 47 | 57 | 62 |
| Blood group of donor | $O^+$ | $A^+$ | $A^+$ |
| Type of liver transplant | *Cadaveric* | *Cadaveric* | *Cadaveric* |
| Time of liver biopsy after transplantation (months) | 61 | 13 | 63 |
| Histological diagnosis | Alcohol induced microvesicular steatosis | Alcohol induced microvesicular steatosis | Alcohol induced steato-hepatitis |

**Table 2: Characteristics of liver transplant patients.**

All liver transplant patients were male. ALD: alcoholic liver disease; HCV: hepatitis C virus.

**Table 3**

| | **Patient 1** | **Patient 2** | **Patient 3** |
|---|---|---|---|
| $Y^+/CD34^+$ cells expressed as a % of total cells per section | 2.51% (0.97%)* | 0.63% (0.24%)* | 2.37% (0.91%)* |
| % of $Y^+/\alpha$-$SMA^+$ cells | 19.9% (7.8%)* | 7.9% (3.03%)* | 26.8% (10.3%)* |
| % of $CD34^+/\alpha$-$SMA^+$ cells | 6.10%** | 5.03%** | 3.95%** |
| % of $Y^+$/$Hepatocyte^+$ cells | 0% | 0% | 0% |
| % of $Y^+$/$AE1/AE3^+$ cells | 0% | 0% | 0% |
| Histological diagnosis | Alcohol induced microvesicular steatosis | Alcohol induced microvesicular steatosis | Alcohol induced steatohepatitis |

**Table 3: Results of combined FISH and immunostaining.**

The mean proportion of $Y^+$ chromosome cells was 38.46% in the male control section and 0.0% in the female control sections. * Counts unadjusted for correction factor 2.6. ** Dual immunohistochemistry data without FISH for Y-chromosome analysis and not requiring correction factor.

**Figure 8**

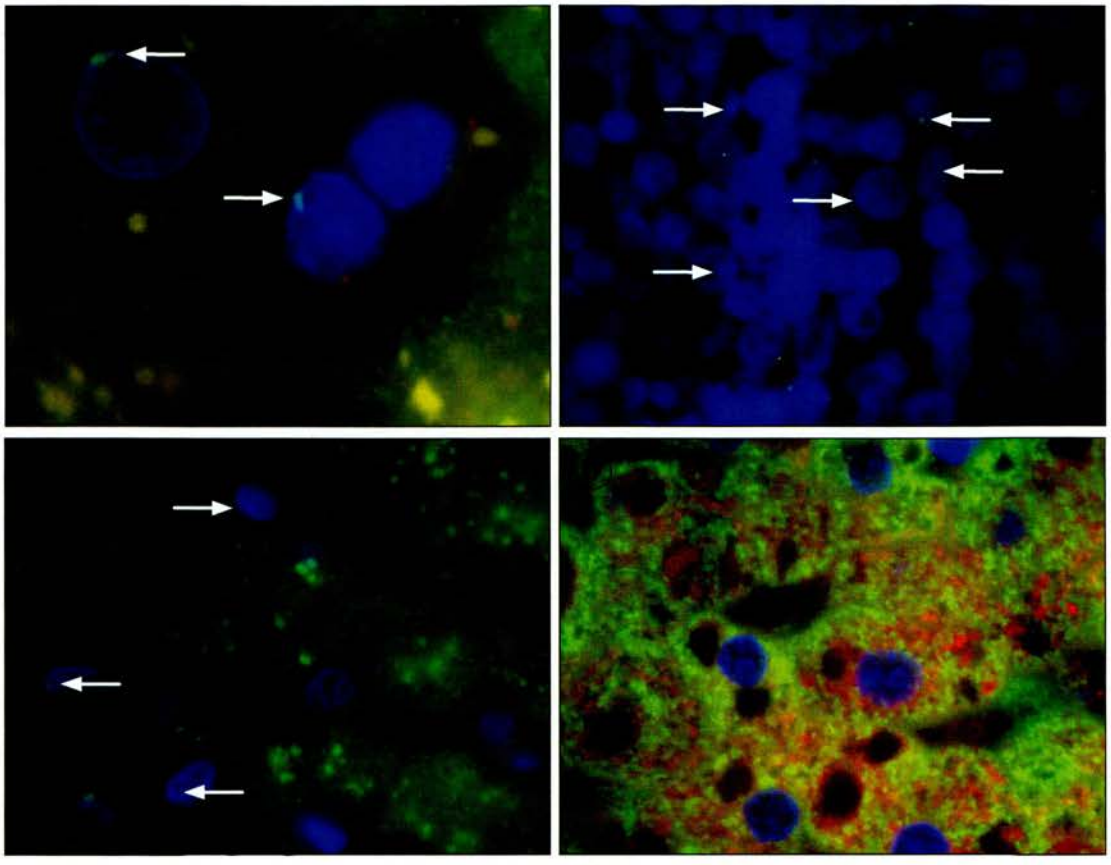

**Figure 8: FISH analysis for y chromosome in liver biopsies from male and female controls.**

**A-C)** Liver biopsies from male controls demonstrating y chromosome with direct *in situ* hybridisation *(green, arrows).* **D)** Liver biopsy from female control demonstrating the absence of y chromosome. Direct *in situ* hybridisation. Original magnification: A-D, 600x.

**Figure 9**

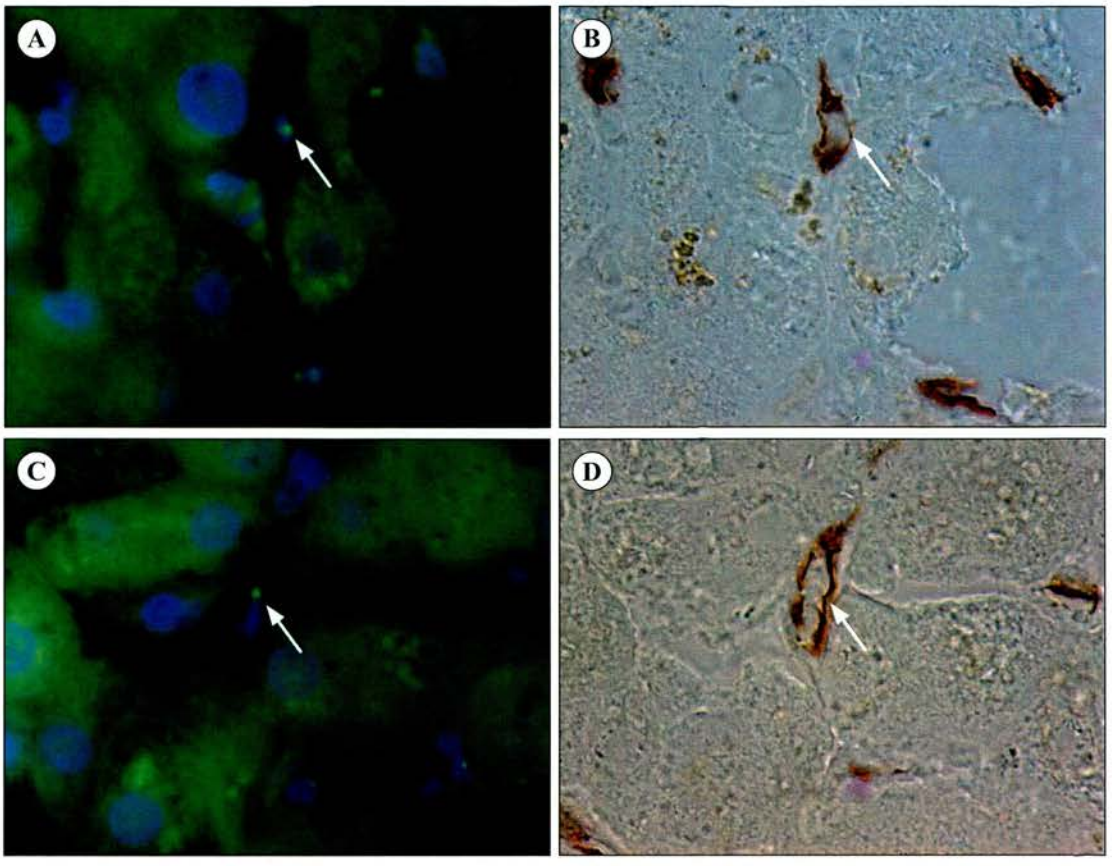


**Figure 9: FISH and immunohistochemical analysis for recipient CD34$^+$ cells in cross-sex donor liver grafts with alcohol injury.**

**A)** Liver biopsy from a male patient transplanted with a female liver and subsequently developed alcohol induced steatohepatitis. Direct *in situ* hybridisation for y chromosome *(green, arrow)*. **B)** Image A combined with immunohistochemistry for CD34 *(brown, arrow)*, CD31 staining was negative. **C-D)** Liver biopsy from a male patient with a cross-sex liver graft and alcohol induced microvesicular steatosis. Direct *in situ* hybridisation for y chromosome *(green, arrow)* combined with immunostaining for CD34 *(brown, arrow)*, CD31 staining was negative. Original magnification: A-D, 600x.

Figure 10

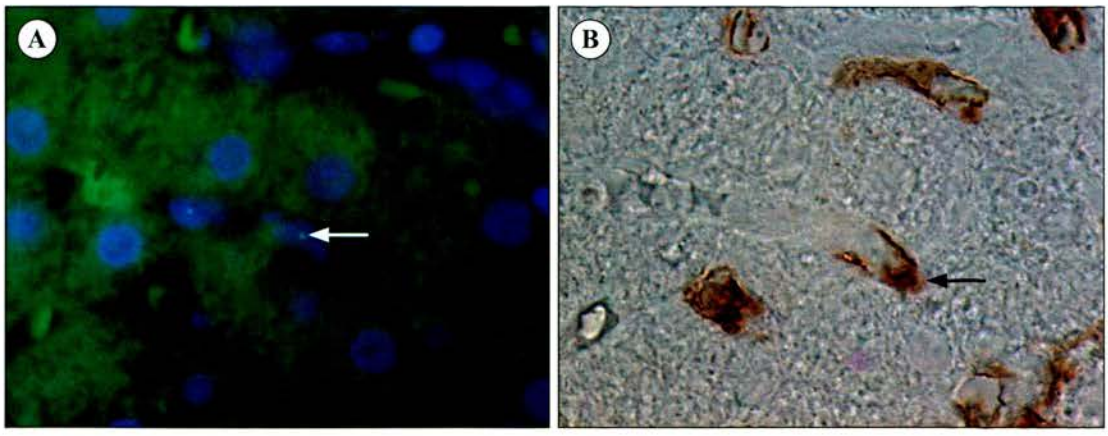


**Figure 10: FISH and immunohistochemical analysis for recipient CD34$^+$ cells in cross-sex donor liver grafts with alcohol injury.**

**A-B)** Liver biopsy from a male patient with a cross-sex liver graft and alcohol induced microvesicular steatosis. Direct *in situ* hybridisation for y chromosome *(green, arrows)* combined with immunostaining for CD34 *(brown, arrows)*, CD31 staining was negative. Original magnification: A-D, 600x.

Figure 11

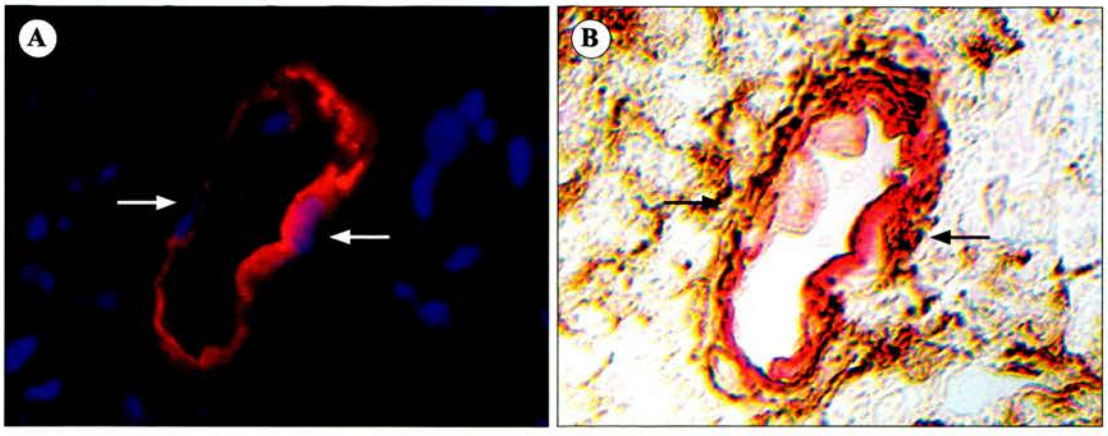


**Figure 11: Immunohistochemistry for CD31$^+$and CD34$^+$ cells in cross-sex donor liver grafts with alcohol injury.**

**A)** Immunofluorescence for CD31 *(red, arrows)* demonstrating hepatic venule CD31$^+$ endothelial cells in a liver section from a male cross-sex liver graft patient with alcohol induced steatohepatitis. **B)** Image B demonstrating co-staining for CD34$^+$ *(brown)* / CD31$^+$ *(red, arrows)*. Original magnification: A-B, 600x.

Figure 12

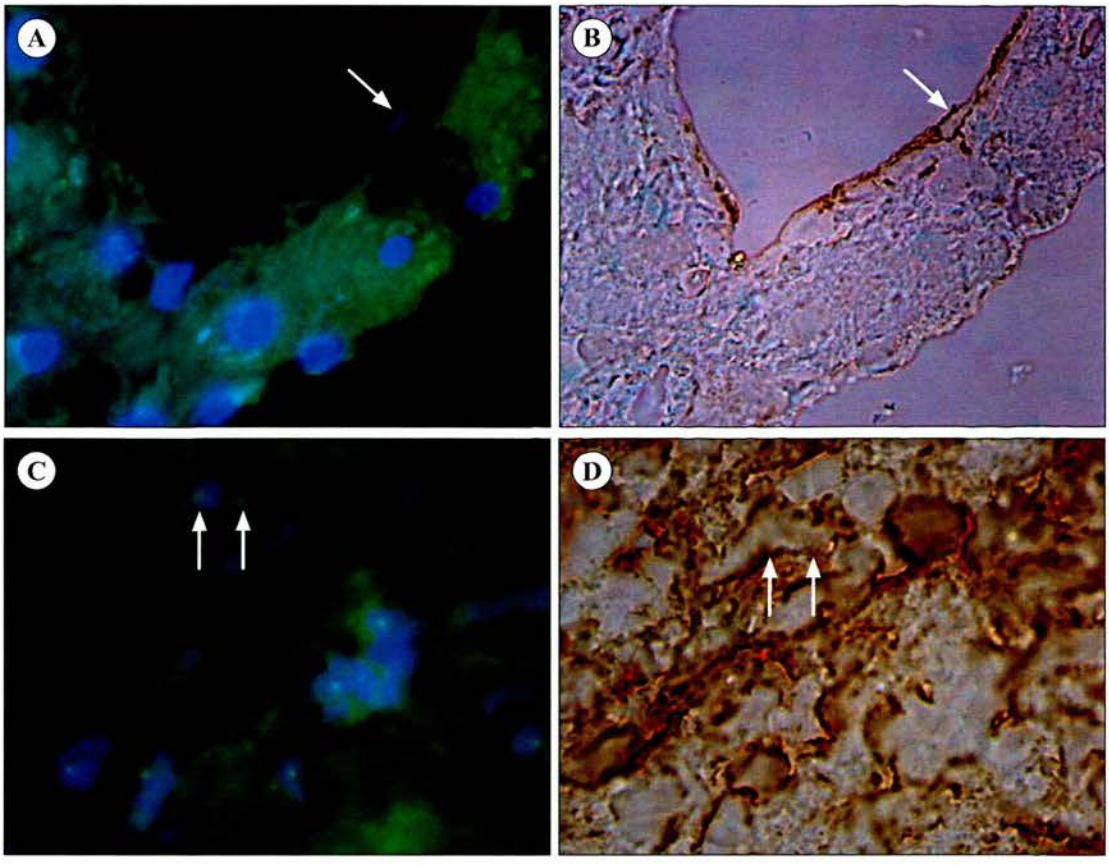


**Figure 12: FISH and immunohistochemical analysis for recipient myofibroblasts (α-SMA$^+$) cells in cross-sex donor liver grafts with alcohol injury.**

**A)** Liver biopsy from a male patient transplanted with a female liver and subsequently developed alcohol induced microvesicular steatosis. Direct *in situ* hybridisation for y chromosome *(green, arrow)*. **B)** Image A combined with immunohistochemistry for α-SMA$^+$ demonstrating recipient derived myofibroblasts *(brown, arrow)*. **C-D)** Liver biopsy from a male patient with a cross-sex liver graft and alcohol induced steatohepatitis. Direct *in situ* hybridisation for y chromosome *(green, arrows)* combined with immunostaining for α-SMA$^+$ *(brown, arrows)*. Original magnification: A-D, 600x.

Figure 13

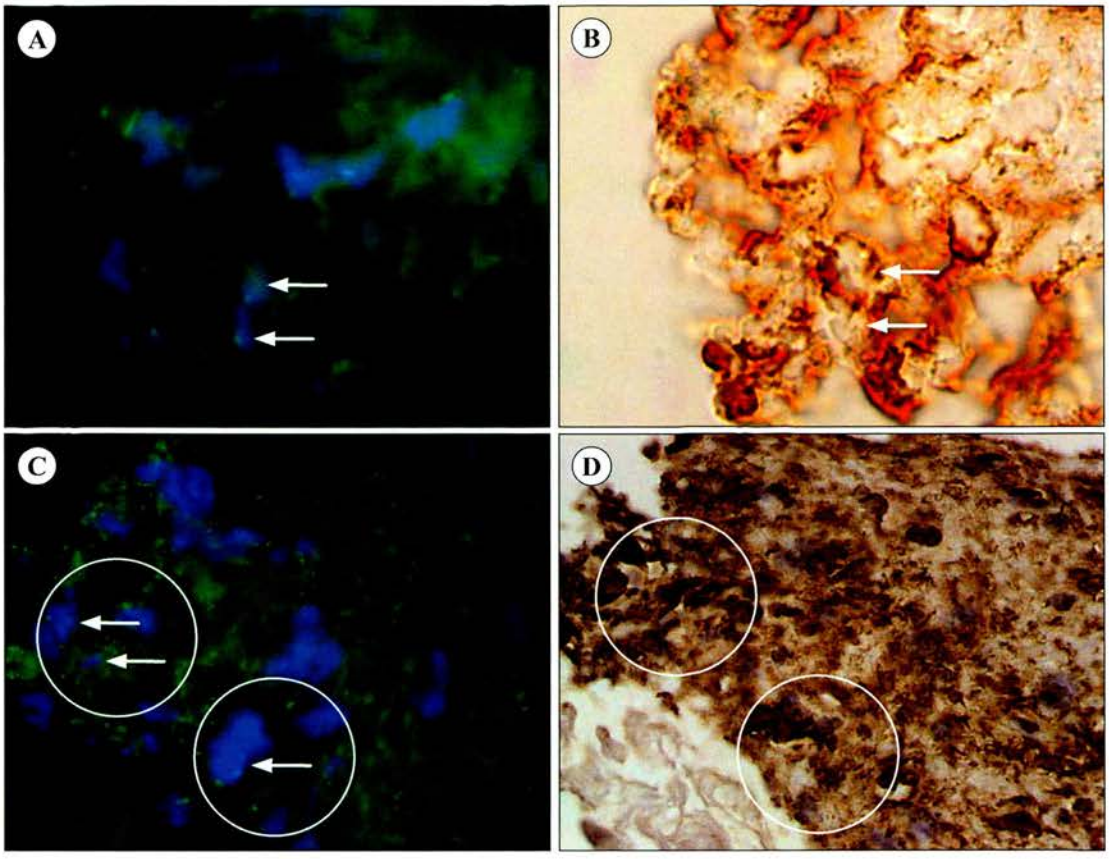


**Figure 13: FISH and immunohistochemical analysis for recipient myofibroblasts (α-SMA$^+$) cells in cross-sex donor liver grafts with alcohol injury.**

**A-B)** Liver biopsy from a male patient with a cross-sex liver graft and alcohol induced steatohepatitis. Direct *in situ* hybridisation for y chromosome *(green, arrows)* combined with immunostaining for α-SMA$^+$ *(brown, arrows)*. **C-D)** 3-μm-thick sequential liver sections from the same male patient with a cross-sex liver graft and alcohol induced steatohepatitis demonstrating recipient y chromosome *(green, arrows and circles)* in the same region as immunostaining for α-SMA$^+$ *(brown, circles)*. Original magnification: A-B, 600x, C-D, 400x.

**Figure 14**

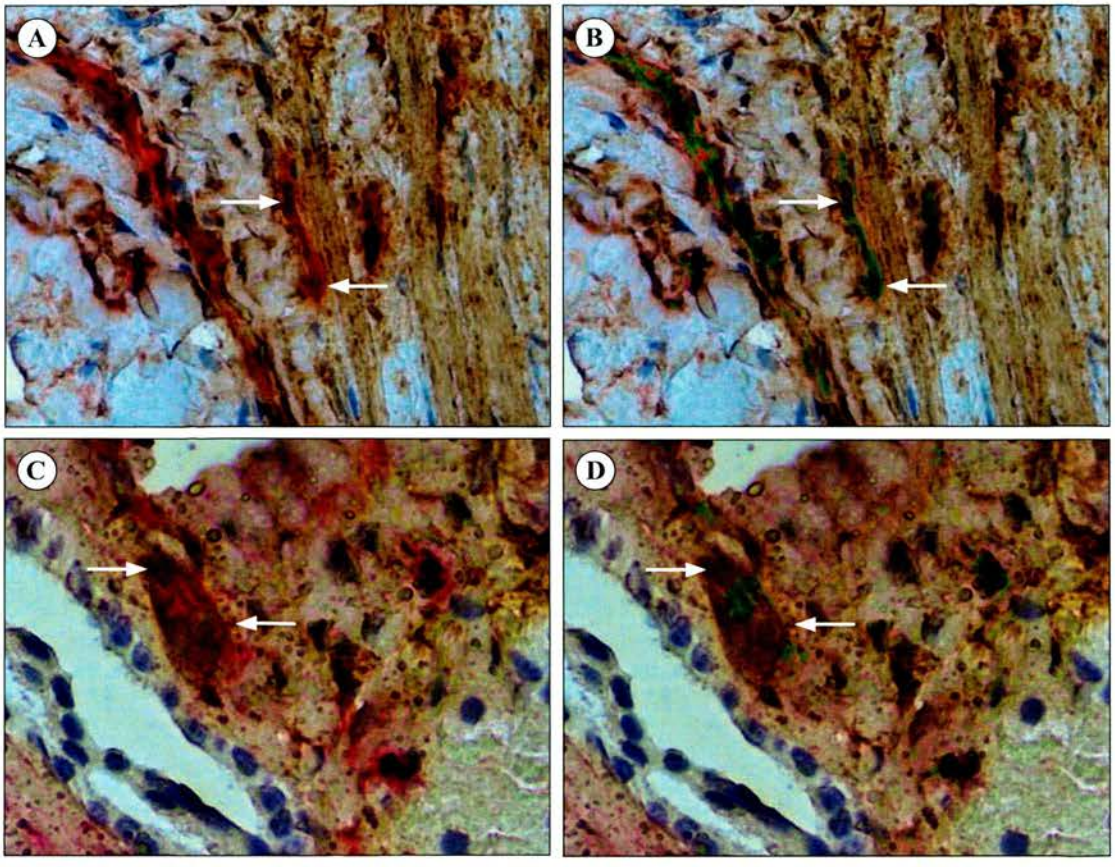


**Figure 14: CD34$^+$ cells are capable of becoming myofibroblasts (α-SMA$^+$) in alcohol induced liver injury.**

**A)** Co-staining for CD34$^+$ *(red)* / α-SMA$^+$ *(brown)* cells in liver section from a male patient with a cross-sex liver graft and alcohol induced steatohepatitis *(arrows)*, note that the CD34$^+$ / α-SMA$^+$ cells are localised within the inflammatory infiltrate and in close proximity to fibrous tissue. **B)** Green pseudo colouring of image A accentuating co-staining for CD34$^+$ *(green)* / α-SMA$^+$ cells *(brown, arrows)*. **C)** Co-staining for CD34$^+$ *(red)* / α-SMA$^+$ *(brown)* cells in liver section from a male patient with a cross-sex liver graft and alcohol induced microvesicular steatosis *(arrows)*. **D)** Green pseudo colouring of image C accentuating co-staining for CD34$^+$ *(green)* / α-SMA$^+$ cells *(brown, arrows)*. Original magnification: A-B, 200x; C-D, 400x. These images have been magnified to demonstrate myofibroblast morphology.

**Figure 15**

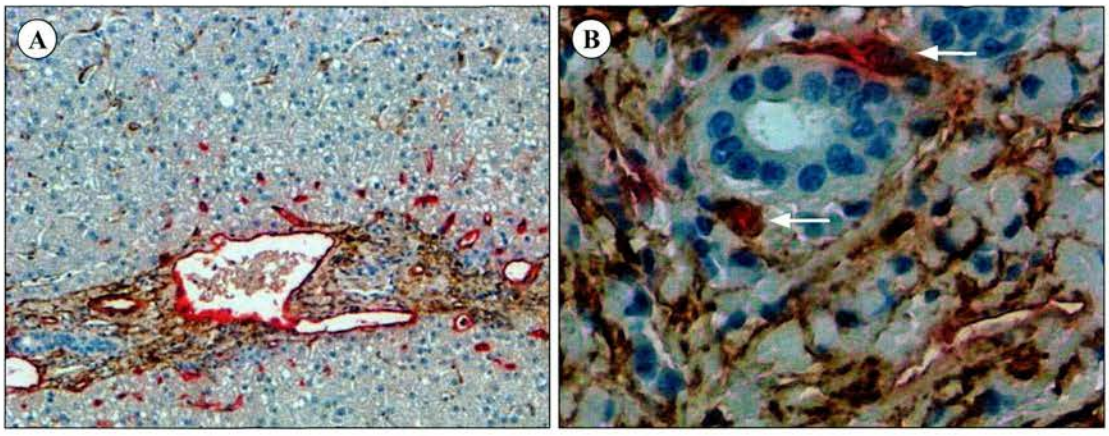


**Figure 15: Immunohistochemical analysis for CD34$^+$/α-SMA$^+$ cells in day 7 post cross-sex liver transplant biopsies *(control)*.**

**A)** Co-staining for CD34$^+$ *(red)* / α-SMA$^+$ *(brown)* cells in liver section from a male patient with a cross-sex liver graft. **B)** CD34$^+$ *(red)* / α-SMA$^+$ *(brown)* cells identified in peri-portal region of liver biopsy from a male patient with a cross-sex liver graft *(arrows)*. Original magnification: A, 50x; B, 400x. Image B has been magnified to demonstrate CD34$^+$/α-SMA$^+$ cell morphology.

Figure 16

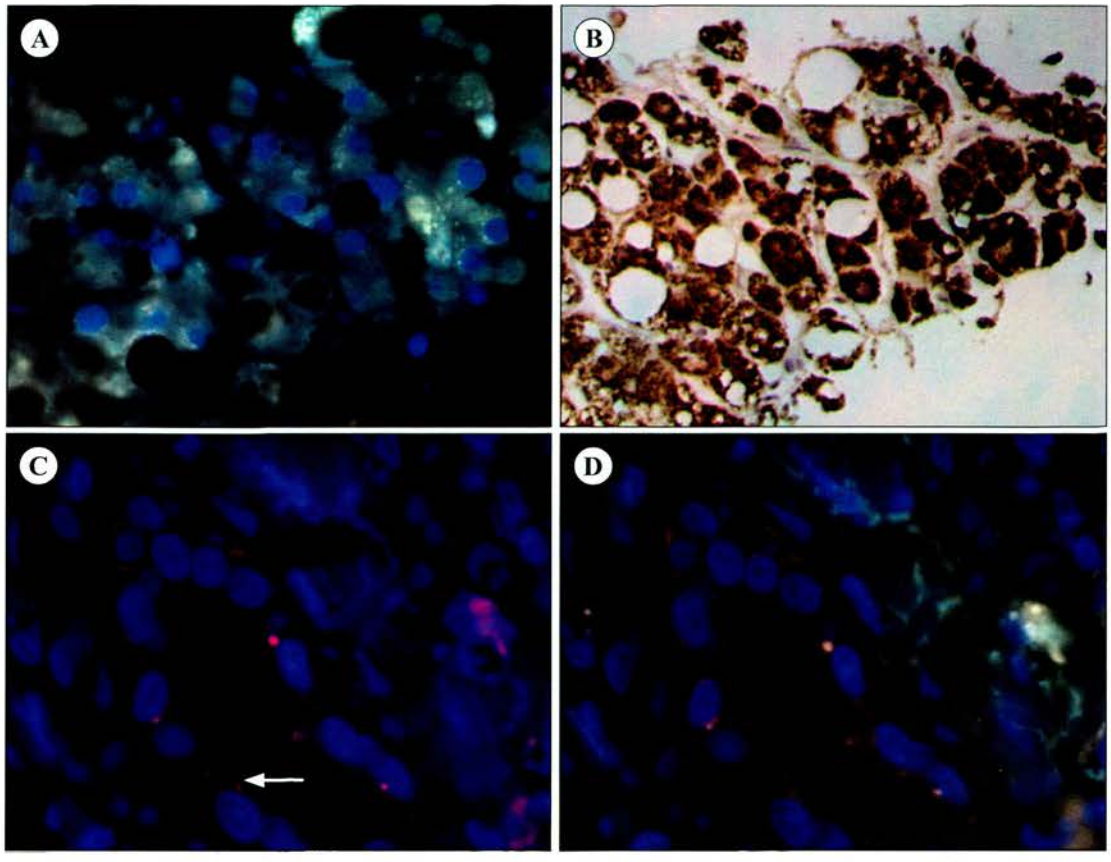


**Figure 16: Recipient hepatocytes and biliary epithelial cells (CK AE1/AE3) were not identified in cross-sex donor liver grafts with alcohol injury.**

**A)** Liver section from a male patient transplanted with a female liver and subsequently developed alcohol induced steatohepatitis. Recipient y chromosome was not detected by direct *in situ* hybridisation. **B)** 3-µm-thick sequential liver section from the same male patient in image A stained for human hepatocyte marker Hep Par 1 *(brown)*. Both image A and B represent the same region of biopsy tissue. **C)** Liver biopsy from a male patient with a cross-sex liver graft and alcohol induced steatohepatitis stained for biliary marker CK AE1/AE3 *(red, arrow)*. **D)** Liver section from a male patient transplanted with a female liver and subsequently developed alcohol induced steatohepatitis. Recipient y chromosome was not detected in the biliary epithelium by direct *in situ* hybridisation combined with immunostaining for CK AE1/AE3 *(red)*. Original magnification: A-B, x400; C-D, x600.

**Figure 17**

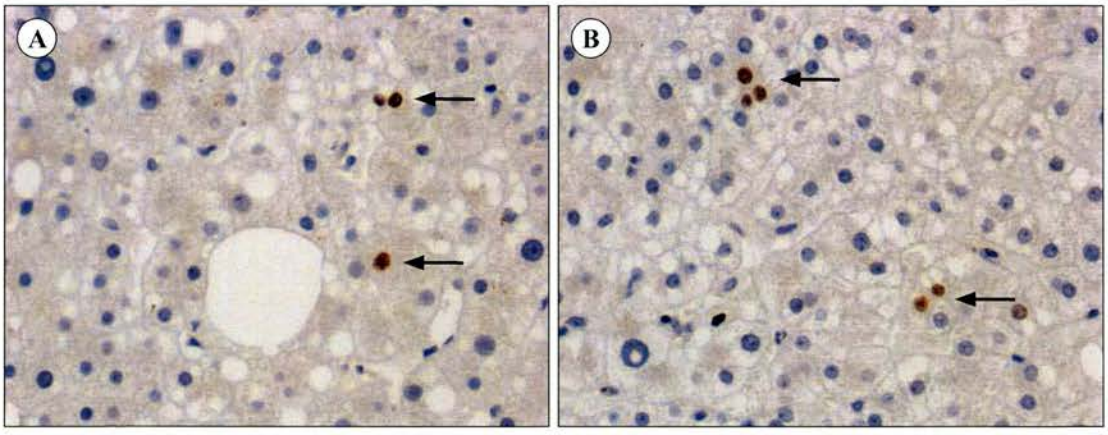


**Figure 17: Immunostaining for Ki-67 in cross-sex donor liver grafts with alcohol injury.**

**A-B)** Liver section from a male patient transplanted with a female liver and subsequently developed alcohol induced steatosis. Staining for Ki-67 indicates hepatic parenchymal cell proliferation *(brown, arrows)*. Original magnification: A-B, x400.

**Figure 18**

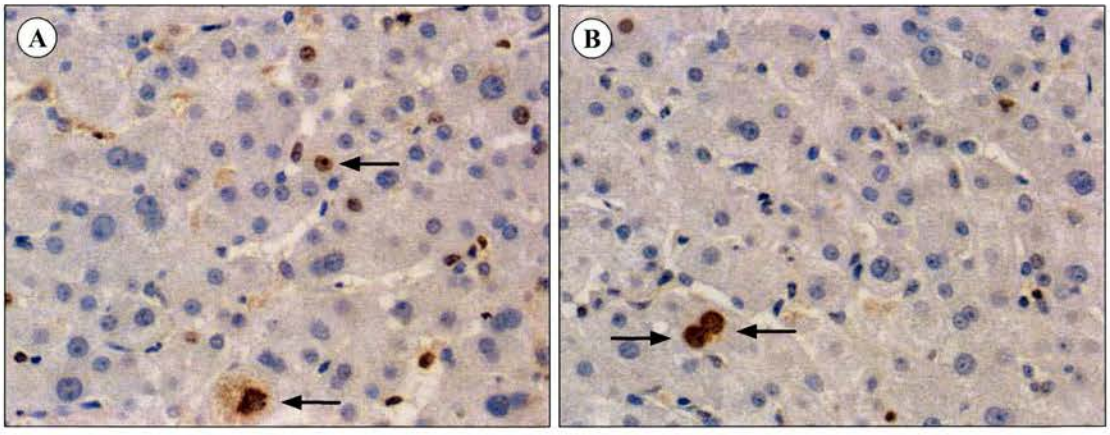


**Figure 18: Immunohistochemical analysis for Ki-67 in day 7 post cross-sex liver transplant biopsies *(control)*.**

**A-B)** Liver section from a male patient transplanted with a female liver. Staining for Ki-67 indicates hepatic parenchymal cell proliferation *(brown, arrows)*. Original magnification: A-B, x400.

# CHAPTER 4

## Mobilisation of circulating haematopoietic stem cells in human alcohol induced liver injury

## 4.1 Introduction

In the previous chapter **(Chapter 3)** we demonstrated that human BM derived HSCs are recruited into the alcohol injured liver. As part of this recruitment process, we aim in this chapter **(Chapter 4)** to establish that alcohol induced liver injury mobilises human bone marrow derived HSCs into the peripheral circulation without altering their stem cell potential. Previous human studies have demonstrated increased levels of circulating human HSCs in response to a systemic injury, surgical trauma and in patients following extensive liver resection (Grzelak et al. 1998; Lamming et al. 2003; Paczkowska et al. 2005; De Silvestro et al. 2004). The extent to which these peripheral blood HSCs are mobilised into the circulation of patients with other types of liver injury in particular alcohol induced injury has not been well studied.

The chemokine SDF-1 and its receptor CXCR4, play an important role in the mobilisation of human and rodent HSCs from BM both in the absence and presence of liver injury (Kollet et al. 2003; Lapidot & Petit 2002; Petit et al. 2002; Terada et al. 2003). Their role in clinical liver injury however has not been studied. The chemokines MIG and IP-10, which mediate their effect through chemokine receptor CXCR3, are important in recruiting T lymphocytes into the injured liver as well as playing a crucial role in $CD34^+$ HSC adhesion and therefore could also be important in the recruitment of HSCs in liver disease (Jinquan et al. 2000; Nanji et al. 1999).

In this chapter we wanted to establish if the CXCR3 and CXCR4 chemokine receptor profiles of circulating HSCs are important in regulating the mobilisation process in alcohol induced liver injury.

## 4.2 Patients and methods

### 4.2.1 Patient details

In this study, blood samples from patients with AH were used to establish whether human BM derived HSCs are mobilised into the peripheral circulation in alcohol induced liver injury. Blood samples were collected from healthy volunteer controls and from patients attending the Royal Infirmary of Edinburgh from October 2002 to June 2004 and gave informed consent to use the blood samples for experimental purposes. Ethical approval for the study was granted by the Lothian Health Board Research and Ethics Committee (LREC/2000/4/187). Patients with AH, chronic abstinent alcoholic cirrhosis, primary biliary cirrhosis (PBC), untreated hepatitis C virus (HCV) and acute paracetamol injury were included in this study. Blood samples from AH patients were taken within the first week of admission and for acute paracetamol injury patients taken within 76 hours of admission. All other patient blood samples were taken at routine out patient clinic visits. Haematological and biochemical laboratory data from all patients were also collected. Blood samples from 15 AH patients (7 Female, 8 Male, Mean age 49yrs), 7 acute paracetamol injury patients (2 Female, 5 Male, Mean age 35yrs), 8 chronic abstinent alcoholic cirrhosis patients (1 Female, 7 Male, Mean age 48yrs), 7 untreated HCV patients (2 Female, 5 Male, Mean age 44yrs), 6 PBC patients (all Female, Mean age 58yrs) and 12 normal healthy volunteers (6 Female, 6 Male, Mean age 31yrs) were obtained. The mean age, male to female ratios, numbers (n) and biochemical parameters for all groups are summarised in ***table 4***.

Blood samples from AH patients were included if they fulfilled the following parameters: 1) Recent alcohol abuse (>400gm/week), 2) Jaundice (serum bilirubin >50μmol/l), 3) one or more of the following criteria: hepatomegaly, pyrexia, leukocytosis and 4) No previous documented history of alcoholic cirrhosis. We did however accept that in this AH patient group there would be a proportion of patients with established alcoholic

cirrhosis on admission even though it may have not been previously documented or identified. Exclusion criteria were concurrent sepsis, corticosteroid treatment, recent gastrointestinal bleeding and significant co-morbidity such as cardiorespiratory failure or neoplasia.

All patients with AH had an admission prognostic formula calculated known as Maddrey's discriminant function (DF) (Maddrey et al. 1978). This formula has been widely used as a tool to assess severity of AH and has been validated in several studies: DF = 4.6 x (PT patient - PT control) (s) + serum bilirubin (μmol/l) / 17.1. Values of more than 32 effectively identify patients whose one-month mortality is 50%. Eight patients with significant AH (Maddrey's DF >32) in this study were all treated with Pentoxifylline, a non-selective phosphodiesterase inhibitor that has been shown to decrease TNF-α gene transcription (Strieter et al. 1988) as well as the production of other cytokines and chemokines including interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ), IL-8, NE, MCP-1 and RANTES (Gutierrez-Reyes et al. 2006;Morgan & McClain 2000a;Neuner et al. 1994;Oka et al. 1991;Seldon et al. 1995).

We did not exclude these patients from our study as it would not only limit our sample size but by doing so our data would be skewed and less representative of the spectrum of severity of AH patients that is seen clinically. Pentoxifylline is not known to directly inhibit HSC mobilisation although it does modify the host immune responses and is likely to have an effect on cytokine levels. We acknowledge this fact as a limitation of the study and will address these limitations further in the discussion **Chapter 6**.

### 4.2.2 Peripheral blood $CD34^+$ cell enumeration and chemokine receptor profile

Peripheral blood samples (40mls) was collected into heparinised syringes and prepared immediately. Light density fraction mononuclear cells were isolated from

the peripheral blood by centrifugation using 1.077g/l Histopaque® solution (Sigma Diagnostics, St Louis, MA, USA, 1077) in a 50ml Leuco Sep tube containing filter (Greiner Bio-One GmbH).

The light density monolayer was separated and incubated with red cell lysis buffer for 5 minutes. The sample was then washed with sterile PBS until visibly clear of erythrocytes. Red cell lysis buffer was prepared with $NH_4Cl$, $NaHCO_3$, disodium EDTA and made up to pH7.4 with NHCl or $Na_2OH$.

Cells were counted with an inverted microscope (Zeiss Telaval 31, Germany) for correct antibody concentrations and the mononuclear cell enriched suspension was labelled with mouse anti-human CD45: R-phycoerythrin- cyanine dye 5 (RPE-CY5) (Serotec Ltd, Oxford, UK, MCA 1719C), mouse anti-human CD34: Fluorescein Isothiocynate (FITC) (BD Biosciences, San Diego, CA, USA, 555821), mouse anti-human CXCR4: phycoerythrin (PE) (R&D Systems Europe Ltd, Oxon, UK, FAB170P) and mouse anti-human CXCR3: PE (R&D Systems Europe Ltd, Oxon, UK, FAB160P) conjugated monoclonal antibodies. The labelled cells were incubated at 4°C for 30 minutes in the dark and then washed twice with PBS (5 minutes at 500g) before being resuspended in 500µl PBS for analysis by flow cytometry.

A reliable marker for circulating haematopoietic stem and progenitor cells has led to the development of the single-platform International Society for Haemotherapy and Graft Engineering (ISHAGE) flow cytometric assay which quantitates such cells on the basis of their expression of CD34 (Sutherland et al. 1996). This single-platform ISHAGE method for $CD34^+$ cell enumeration has been internationally validated and is widely used for evaluation of graft adequacy of peripheral blood and bone marrow stem cell grafts. Using the FACS VANTAGE® flow cytometry sorting machine, $CD34^+$ cells were quantified as a percentage of low side scatter mononuclear cells using ISHAGE

flow cytometry guidelines (Sutherland et al. 1996) which uses forward/side scatter gating followed by gating on the marker CD45 and CD34. The first stage of analysis is to plot the events on a forward scatter *vs.* side scatter dotplot and to select a gate around the low side scatter population of mononuclear cells which predominantly comprise of lymphocytes which excludes debris, platelets, macrophages, monocytes and unlysed erythrocytes. In the second stage, the selected mononuclear cell population is then displayed on a CD45 *vs.* CD34 dotplot and the cluster of $CD34^+$/CD45 *intermediate* events detected are gated. Finally the $CD34^+$/CD45 *intermediate* population selected is then quantified as a percentage of the total events gated in the first stage of analysis i.e. low side scatter mononuclear cells ***(see figure 19)***. The percentage of $CD34^+$ cells expressing CXCR3/CXCR4 as well as CXCR3/CXCR4 receptor density was also quantified by flow cytometry.

### 4.2.3 Colony forming unit cell assays

To prepare cells for culture, Iscoves Modified Dulbecco's Media (IMDM) (Stem Cell Technologies, Vancouver, BC, Canada, 07700) was used for all washes and dilutions instead of PBS. After $CD34^+$/$CD45^+$ cell quantification as described above, the $CD34^+$/$CD45^+$ cells from AH patients and NC were sorted using the FACS VANTAGE® flow cytometry sorting machine and collected into IMDM containing Eppendorf tubes at a concentration of $4.5 \times 10^3$ cells/mL. Cells were added to a methylcellulose culture tube, consisting of 1% methylcellulose in IMDM, 30% foetal bone serum, 1% bovine serum albumin, $10^{-4}$M 2-mercapoethanol, 2mM L-glutamine, 50ng/ml stem cell factor, 10ng/ml GM-CSF, 10ng/ml IL-3, 3 units/ml erythropoietin (Methocult™, StemCell Technologies, Vancouver, BC, Canada, GF H4434) and vortexed. With a syringe and blunt ended needle, 2.5ml of this solution was drawn up and 1.1ml added into two 40mm x 10mm Petri dishes (StemCell Technologies Inc. Vancouver, BC, Canada), producing a final cell culture concentration of 450 cells per plate. All cultures were performed in duplicate and incubated at 37°C in a 95% humidified 5% $CO_2$ atmosphere

for 14 days at which point all cell cultures would be terminally differentiated. Burst-forming unit-erythroid (BFU-E), colony-forming unit granulocyte, macrophage (CFU-GM) and colony-forming unit granulocyte,erythroid, macrophage,megakaryocyte (CFU-GEMM) colonies together referred to as colony forming units (CFUs) were counted after 14 days using a phase contrast inverted light microscope (Zeiss Telaval 31, Germany). In order to identify the possibilty that other progenitor cells different from the $CD34^{+}/CD45^{+}$ identified in the AH and NC groups were also present in the circulating blood we collected corresponding $CD34^{-}/CD45^{+}$ cell fractions and grew them using the same culture conditions.

### 4.2.4 Statistics

All statistics were performed on SPSS® 12.0 for Windows® software (SPSS Inc. Chicago, IL, USA). Results are expressed as the Mean with Standard Error of the Mean (SEM) for individual column data. Differences between groups were analysed with a one way analysis of variance (ANOVA) test with post-hoc Dunnets test to compare each group with normal controls. Results were considered significant when $p<0.05$.

## 4.3 Results

### 4.3.1 Circulating $CD34^+$ HSC levels are elevated in patients with alcoholic hepatitis and retain their stem cell properties

No significant difference in the sex or age distribution was demonstrated between the AH and the NC group. Circulating $CD34^+$ HSCs levels were significantly elevated in patients with AH (0.195 ± 0.063%, $p<0.05$) when compared with the NC group (0.067 ± 0.01%) ***(see figure 20)***. There was no significant difference in the $CD34^+$ values for either the acute paracetamol liver injury (0.068 ± 0.013%) or chronic liver injury groups (0.08 ± 0.014%) when compared with the NC ***(see figure 20)***. This would indicate that the rise in circulating $CD34^+$ values was a specific response to AH, rather than a non-specific response to other forms of liver injury. The total lymphocyte counts were not significantly different in the AH group (1.216 ± 0.146 x $10^9$/l) when compared with the NC (1.55 ± 0.095 x $10^9$/l), chronic liver injury (1.317 ± 0.151 x $10^9$/l) and acute paracetamol liver injury groups (0.83 ± 0.182 x $10^9$/l). The circulating neutrophil counts were significantly elevated in AH patients (9.39 ± 1.96 x $10^9$/l, $p<0.05$) as compared with the NC (3.24 ± 0.36 x $10^9$/l) and other types of liver injury. The mean Maddrey score which is a prognostic score of severity for patients with AH, was 52.507 ± 11.225 consistent with clinically severe AH. Patient profile, admission Maddrey Score, peripheral blood $CD34^+$ count and 28 day survival for patients with AH are tabulated in ***table 5***.

Although the mean Maddrey score in the AH patient group was 52.5 and indicative of severe disease (with expected 28 day mortality >50%) only 3 patients (20%) in the AH study cohort were deceased suggesting that this patient group studied was not reflective of the natural history of AH. A possible explanation for this lower than expected mortality rate in our study cohort could be that patients with sepsis, recent gastrointestinal bleeding and significant co-morbidities were excluded and thus omitting

the common causes for morbidity and mortality in such patients with severe AH. We noted that the 12 surviving AH patients had higher levels of circulating $CD34^+$ HSCs ($0.24 \pm 0.08\%$) when compared with the 3 AH patients who had died ($0.07 \pm 0.03\%$). However as patient numbers in the deceased AH group were too small for comparison we could not draw any firm conclusion regarding HSC mobilisation and its effect on the subsequent course of illness in AH patients.

To determine the 'stemness' of these mobilised cells, we performed CFU assays of the $CD34^+$ isolated cells. The CFU frequency per plate (containing $0.45 \times 10^3$ $CD34^+$ cells) in AH and NC are shown in ***figure 21***. In the AH patients there were significantly higher levels of $CD34^+/CD45^+$ total CFU counts as compared with NC HSCs ($154 \pm 38$ *vs.* $44.75 \pm 28.4$, $p<0.05$). Interestingly, there was also a significantly higher level of $CD34^-/CD45^+$ total CFU counts in the AH group as compared with NC ***(see table 6)***. In both patient groups the cell assays demonstrated mature and multiple lineage CFUs ***(see figure 22)***.

### 4.3.2 $CD34^+$ mobilisation in alcoholic hepatitis is not associated with alterations in the cell CXCR3/CXCR4 receptor expression

There was no significant difference in either the percentage of $CD34^+$ expressing CXCR4 or receptor density / cell in the AH group ($86.66 \pm 4.12\%$ & geometric mean [GM] $71.71 \pm 21.52$ respectively) when compared with the NC ($80.36 \pm 6.6\%$ & GM $63.35 \pm 21.4$), acute paracetamol injury ($83.46 \pm 4.14\%$ & GM $56.65 \pm 16.73$) and chronic liver injury groups ($80.08 \pm 2.47\%$ & GM $64.57 \pm 12.07$) ***(see figure 23)***. Furthermore, no significant difference was demonstrated in either the percentage of HSCs expressing CXCR3 or receptor density / cell in the AH group ($82.9 \pm 5.13\%$ & GM $57.9 \pm 20.6$ respectively) when compared with NC ($70.18 \pm 9.4\%$ & GM $37.0 \pm 8.61$) acute paracetamol injury ($73.32 \pm 7.12\%$ & GM $53.87 \pm 12.43$) and chronic liver injury groups ($70.635 \pm 3.995\%$ & GM $45.178 \pm 8.453$) ***(see figure 24)***.

Although no significant difference was identified in CXCR3 and CXCR4 receptor expression on circulating HSCs, their functionality was not assessed. It is possible that the CXCR3 and CXCR4 receptor function on circulating $CD34^+$ cells could have been altered in the all types of liver injury despite any changes seen in receptor expression. We did not pursue CXCR3 or CXCR4 receptor functional experiments such as Boyden chamber chemotaxis assays and/or intracellular free calcium fluorimetry assays due to the limited number of $CD34^+$ cells available from each sample to do these assays.

Eventhough we identified a significant increase in circulating $CD34^+$ HSCs in AH patients, the relative concentration of circulating $CD34^+$ cells in peripheral blood is still low ranging from a mean of 0.067% in NC to 0.195% in AH patients. At these low concentrations, a 40ml peripheral blood sample (the maximum amount approved by ethics) would only yield between $5 \times 10^3$ to $11 \times 10^3$ $CD34^+$ cells when collected using the FACS VANTAGE® flow cytometry sorting machine. The cell culture assays alone required $4.5 \times 10^3$ cells/mL per sample, thus leaving no surplus cells available for us to perform the functional assays which required a minimum of $24 \times 10^3$ for the Boyden chamber chemotaxis assays and $40 \times 10^3$ for the intracellular free calcium fluorimetry assays.

## 4.4 Conclusion

Our study demonstrated that in patients with acute alcoholic hepatitis circulating $CD34^+$ HSCs levels were elevated as a response to liver injury and retained their true stem cell properties. We failed to demonstrate a similar significant elevation in circulating $CD34^+$ cell counts in other acute or chronic liver injury groups studied. The CXCR3 and CXCR4 receptor expression/profiles were unaltered on these circulating $CD34^+$ stem cells although cell surface receptor functionality was not assessed. All blood samples were non serial time course samples and patients with severe AH were treated with Pentoxifylline which may have an influence on the results.

**Table 4**

| | **Alcoholic Hepatitis** | **Acute Paracetamol Injury** | **Chronic Liver Injury of variable aetiology** | **Normal Controls** |
|---|---|---|---|---|
| Mean Age in years | 49 ± 3.06 | 35.29 ± 3.73 | 50 ± 2.10 | 31.58 ± 3.16 |
| Male:Female Ratio | 1:0.875 | 1:0.4 | 1:0.75 | 1:0.875 |
| Group Numbers | 15 | 7 | 21<br>(HCV =7)<br>(PBC =6)<br>(AAC =8) | 12 |
| Serum bilirubin concentration (μmol/l)* | 288.33 ±59.16 | 114.14 ±23.57 | 46.95 ±12.04 | 20.2 ±4.88 |
| Serum ALT concentration (IU/l)* | 53.87 ±10.01 | 4610.86 ±568.15 | 50.1 ±8.13 | 24.36 ±4.23 |
| Serum albumin concentration (g/l)* | 28.47 ±1.04 | 31 ±1.90 | 37.05 ±1.81 | 46 ±1.62 |
| Prothromin time (seconds)* | 21.13 ±7.15 | 35.14 ±4.19 | 11.93 ±0.68 | N/A |

**Table 4: Patient characteristics and serum biochemical parameters of peripheral blood sample groups.**

*Data represents mean values for each group ± SEM. AAC: abstinent alcoholic cirrhosis; PBC: primary biliary cirrhosis; HCV: hepatitis C virus; ALT: alanine-amino transferase; N/A: not available.

**Table 5**

| Pt | Age (yrs) | Gender | Admission Maddrey Score | $CD34^+$ count (as % of mononuclear cells) | Neutrophil count ($x10^9/l$) | Lymphocyte count ($x10^9/l$) | Total $CD34^+$ count ($x10^7/l$)* | Outcome -28 Day survival | Pentoxifylline Treatment | Blood Sample Day |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 45 | F | 28.6 | 0.11 | 12.09 | 0.49 | 0.05 | D | Y | 7 |
| 2 | 46 | M | 49.4 | 0.3 | 10.65 | 1.25 | 0.38 | A | N | 2 |
| 3 | 53 | M | 39.5 | 0.56 | 5.11 | 1.8 | 1.01 | A | N | 7 |
| 4 | 45 | M | 12.3 | 0.23 | 6.28 | 1.21 | 0.28 | A | N | 5 |
| 5 | 40 | F | 19.8 | 0.12 | 7.23 | 2.15 | 0.26 | A | N | 3 |
| 6 | 73 | M | 101.7 | 0.01 | 9.62 | 0.61 | 0.01 | D | Y | 3 |
| 7 | 32 | M | 81.5 | 0.04 | 18.97 | 0.82 | 0.03 | D | Y | 7 |
| 8 | 45 | F | 32.9 | 0.05 | 7.52 | 2.2 | 0.11 | A | N | 4 |
| 9 | 40 | M | 33.25 | 0.16 | 14.55 | 0.68 | 0.11 | A | Y | 6 |
| 10 | 43 | F | 49.9 | 0.18 | 26.97 | 1.55 | 0.28 | A | Y | 7 |
| 11 | 47 | F | 64.36 | 0.10 | 5.8 | 0.73 | 0.07 | A | Y | 7 |
| 12 | 66 | F | 36.8 | 0.08 | 5.74 | 1.32 | 0.11 | A | N | 1 |
| 13 | 55 | F | 15 | 0.02 | 8.85 | 1.75 | 0.04 | A | N | 2 |
| 14 | 68 | M | 39.6 | 0.04 | 4.61 | 0.97 | 0.04 | A | Y | 6 |
| 15 | 37 | M | 183 | 0.93 | 6.72 | 0.71 | 0.66 | A | Y | 4 |

**Table 5: Data profile for patients with alcoholic hepatitis.**

*Total $CD34^+$ numbers are adjusted from the total mononuclear cell counts (lymphocytes) multiplied by $CD34^+$ count (as % of mononuclear cells). M: male; F: female; A: alive; D: deceased; Y: yes; N: no.

**Table 6**

| Group | CFU $CD34^+/CD45^+$ | | | | CFU $CD34^-/CD45^+$ | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | SD | No. | Mean | SEM | SD | No. |
| Normal | 44.75 | 28.42 | 56.85 | 4 | 1 | 0.71 | 1.41 | 4 |
| Alcoholic hepatitis | 153.75* | 38.36 | 76.73 | 4 | 41* | 23.69 | 47.37 | 4 |

**Table 6: Total CFU frequency of $CD34^+/CD45^+$ cells and $CD34^-/CD45^+$ cells were increased in the AH groups as compared with the NC groups.**

Each plate containing 0.45 x $10^3$ cells. SEM: standard error of mean; SD: standard deviation.*$p<0.05$ compared to normal value.

**Figure 19**

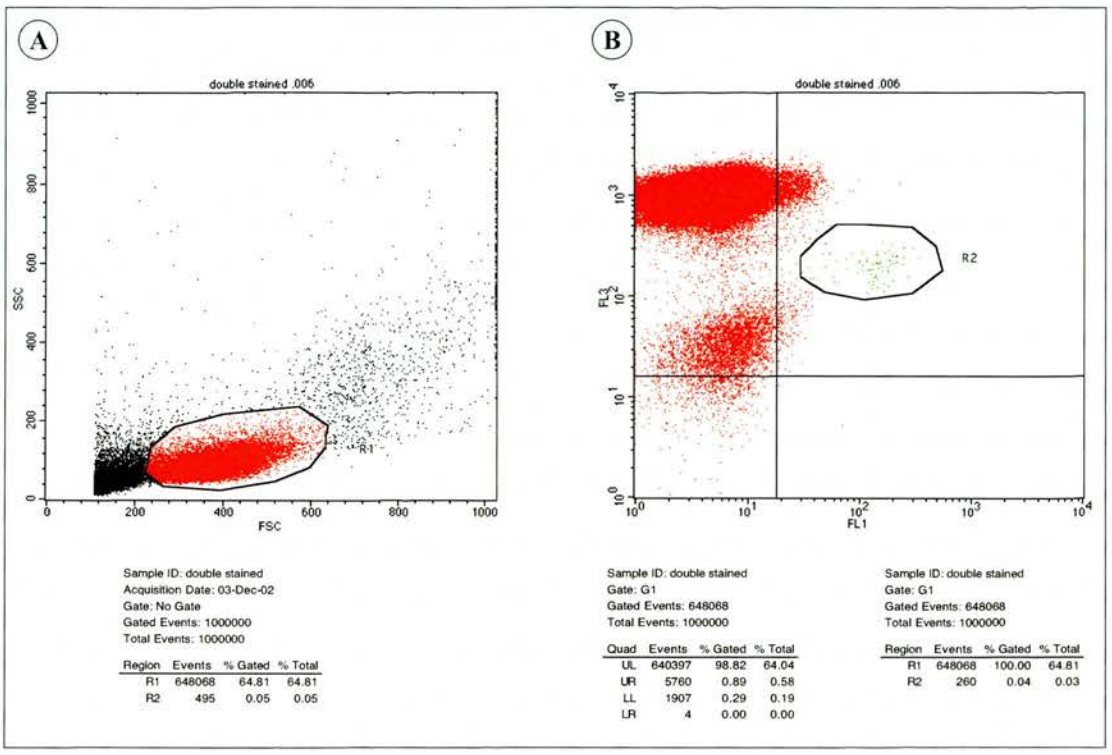


**Figure 19: Circulating CD34$^+$ cell enumeration using flow cytometry.**

**A)** Forward scatter *vs* side scatter dotplot with a selected gate around the low side scatter population of mononuclear cells (R1) which predominantly comprise of lymphocytes. **B)** The selected mononuclear cell population is then displayed on a CD45 *vs* CD34 dotplot and the cluster of CD34$^+$/CD45 *intermediate* events detected are gated (R2). The CD34$^+$/CD45 *intermediate* population selected (R2) is quantified as a percentage of the total events gated in the first stage of analysis i.e. low side scatter mononuclear cells.

Figure 20

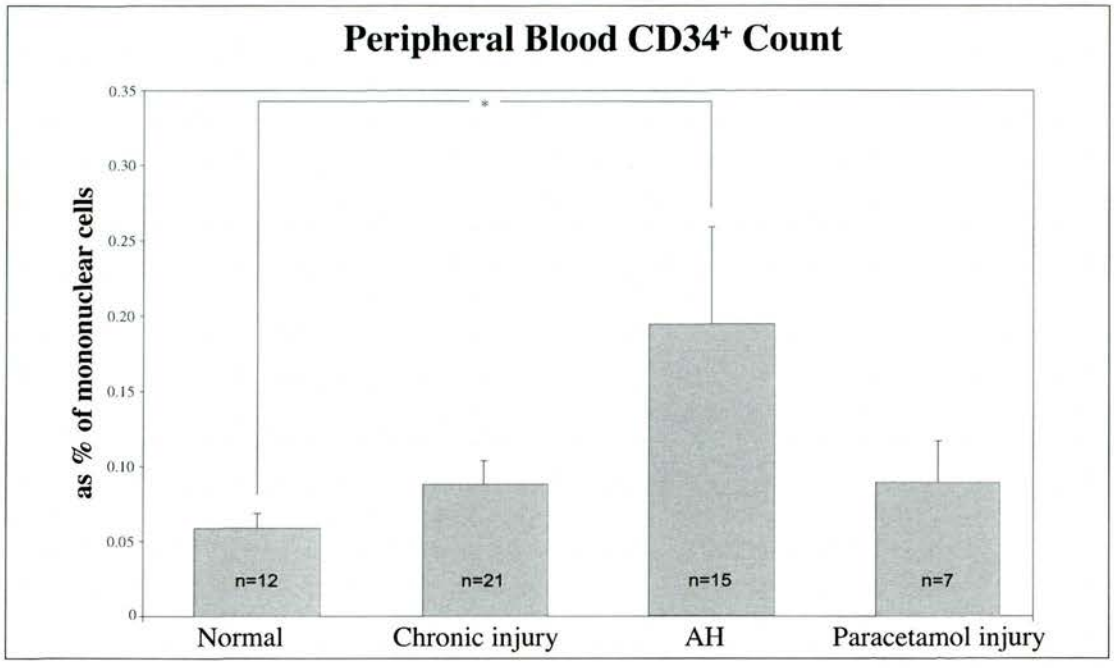


**Figure 20: Peripheral blood CD34$^+$ count is significantly increased in patients with AH compared to NC and other forms of acute or chronic liver injury.**

Values are expressed as a percentage of mononuclear cells present in the serum sample. Data represents mean values for each group ± SEM. N values represents the number of samples in each group. *$p<0.05$ compared to normal value.

**Figure 21**

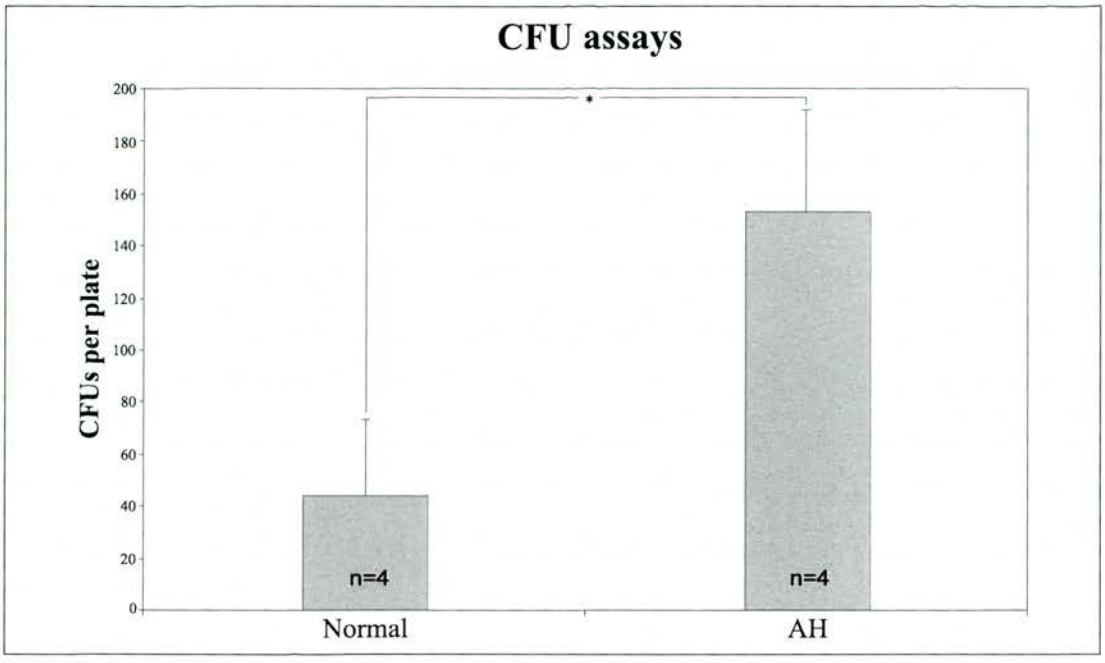


**Figure 21: Peripheral blood CD34$^+$ CFU counts are significantly elevated in AH patients compared to normal controls.**

Values are expressed as CFU's counted per plate. Data represents mean values for each group ± SEM. *$p<0.05$ compared to normal values.

**Figure 22**

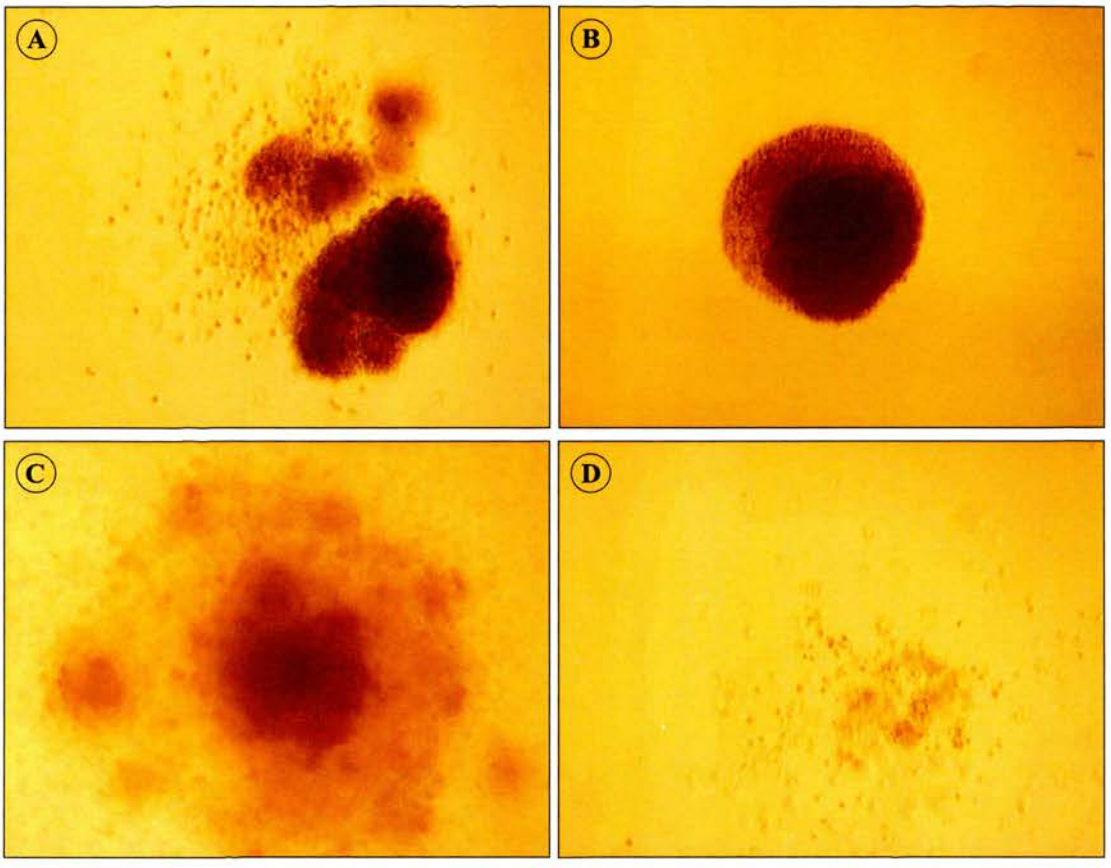


**Figure 22: Images of CFUs obtained from $CD34^+/CD45^+$ stem cell culture assays.**

**A)** Multilineage CFU-GEMM (granulocyte, erythrocyte, macrophage and megakaryocyte). **B-C)** Large erythroid colony BFU (burst-forming unit). **D)** Granulopoietic colonies of CFU-GM (granulocytes and macrophages). Original magnification: A-D, 60x.

**Figure 23**

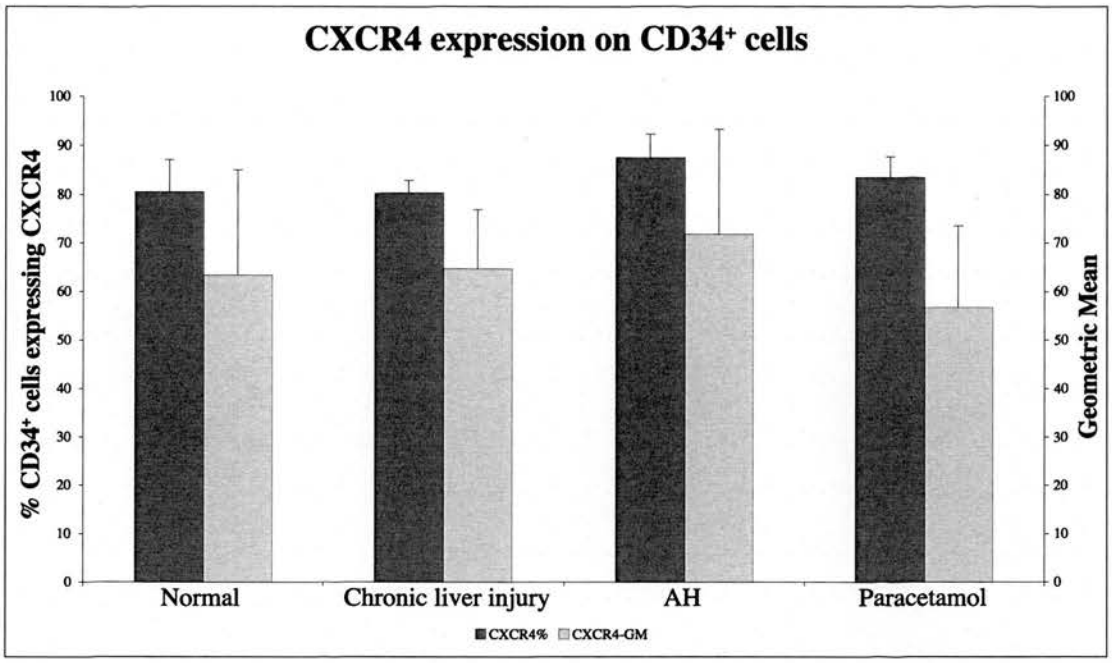


**Figure 23: No significant difference in CXCR4 expression on peripheral blood CD34$^+$ cells in all liver injury groups.**

Values are expressed as a percentage of CD34$^+$ cells expressing CXCR4 and Geometric Mean (GM) of CXCR4. Data represents mean values for each group ± SEM.

**Figure 24**

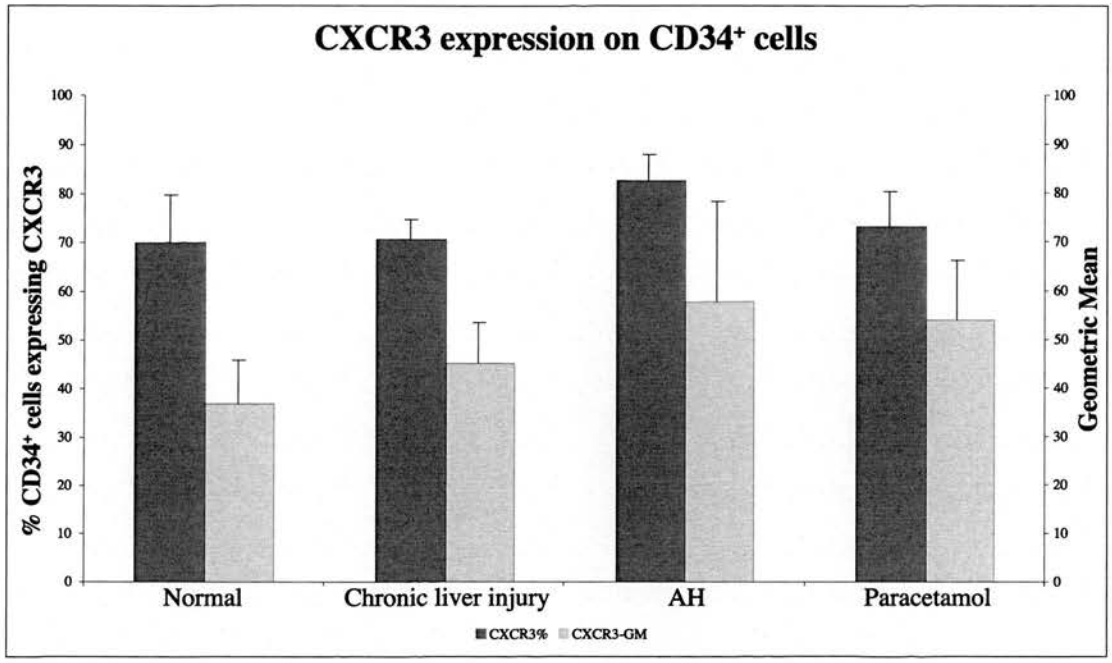


**Figure 24: No significant difference in CXCR3 expression on peripheral blood CD34$^+$ cells in all liver injury groups.**

Values are expressed as a percentage of CD34$^+$ cells expressing CXCR3 and Geometric Mean (GM) of CXCR3. Data represents mean values for each group ± SEM.

# CHAPTER 5

## Inflammatory cytokines and chemokines regulating hepatic recruitment of HSCs in human alcohol induced liver injury

## 5.1 Introduction

Murine and human studies have shown that the chemokine SDF-1 plays an important role in the mobilisation of HSCs from the BM, both in the absence and presence of liver injury and is involved in recruiting HSCs into injured livers and induces the proliferation of endogenous HOCs (Kollet et al. 2003; Lapidot & Petit 2002; Petit et al. 2002; Terada et al. 2003). Other cytokines such as MMP-9, G-CSF, IL-8, NE, MIG, IP-10, RANTES and MCP-1 have also been implicated in the mobilisation and hepatic migration of HSCs although their role in the mobilisation and recruitment of HSC in clinical liver injury has not been defined. In this chapter **(Chapter 5)** we aim to study the role of these inflammatory cytokines and chemokine axes in the mobilisation and recruitment of HSCs in alcohol induced liver injury.

## 5.2 Patient and methods

### 5.2.1 Patient details and sample collection for serum chemokine analysis

The patient cohort that was studied for the mobilisation of HSCs in peripheral blood (Chapter 4) were also used for the serum chemokine analysis study. Serum was collected from peripheral blood samples of healthy volunteer controls and from patients attending the Royal Infirmary of Edinburgh from October 2002 to June 2004. Ethical approval for the study was granted by the Lothian Health Board Research and Ethics Committee (LREC/2000/4/187) and patients gave informed consent to use the blood samples for experimental purposes. Patients with AH, chronic abstinent alcoholic cirrhosis, primary biliary cirrhosis (PBC), untreated hepatitis C virus (HCV) and acute paracetamol injury were included in this study. Blood samples from AH patients were taken within the first week of admission and for acute paracetamol injury patients taken within 76 hours of admission. All other patient blood samples were taken at routine out patient clinic visits.

Blood samples were obtained from 8 normal healthy volunteers (2 Female, 6 Male, Mean age 28yrs), 11 AH patients (6 Female, 5 Male, Mean age 49yrs), 5 acute paracetamol injury patients (1 Female, 4 Male, Mean age 36yrs), and 20 chronic liver injury patients comprising of: 9 chronic abstinent alcoholic cirrhosis patients (1 Female, 8 Male, Mean age 48yrs), 5 untreated HCV patients (2 Female, 3 Male, Mean age 43yrs) and 6 PBC patients (all Female, Mean age 58yrs). Patient characteristics and biochemical parameters for all sample groups are summarised in ***table 7***.

Blood samples from AH patients were included if they fulfilled the following parameters: 1) Recent alcohol abuse (>400gm/week), 2) Jaundice (serum bilirubin >50μmol/l), 3) one or more of the following criteria: hepatomegaly, pyrexia, leukocytosis and 4) No previous documented history of alcoholic cirrhosis. We did however accept that in this AH patient group there would be a proportion of patients with established alcoholic

cirrhosis on admission even though it may have not been previously documented or identified. Exclusion criteria were concurrent sepsis, corticosteroid treatment, recent gastrointestinal bleeding and significant co-morbidity such as cardiorespiratory failure or neoplasia. Seven out of the eleven patients with AH (64%) included in this study were treated with Pentoxifylline which has been shown to decrease TNF-α gene transcription as well as the production of other cytokines and chemokines including IL-1β, IL-6, IFN-γ, IL-8, NE, MCP-1 and RANTES (Gutierrez-Reyes et al. 2006;Morgan & McClain 2000a; Neuner et al. 1994;Oka et al. 1991;Seldon et al. 1995). All 7 AH patients treated with Pentoxifylline had significant AH with a Maddrey's DF >32. We acknowledge this as a limitation of the study and will address these limitations further in the discussion Chapter 6. Currently there no published evidence to suggest that Pentoxifylline has any effect on SDF-1, MMP-9, G-CSF, MIG or IP-10 levels.

Peripheral blood samples (10mls) were collected into unheparinised beaded tubes and allowed to clot for 30 minutes followed by 10 minutes centrifugation at 3000g. The serum layer was separated by pipette into 0.5ml eppendorf tube aliquots and stored in the dark at -20°C. Samples were thawed to 4°C prior to analysis and used once only to avoid repeated freeze-thaw cycles of the serum samples.

### 5.2.2 Serum SDF-1, MMP-9, G-CSF and neutrophil elastase analysis

Serum levels of SDF-1, MMP-9, G-CSF and neutrophil elastase (NE) were measured by enzyme linked immunosorbent assay (ELISA) using the Quantikine® immunoassay kit for human SDF-1α, MMP-9 (total), G-CSF (R&D Systems Europe Ltd, Oxon, UK, DSA00, DMP900, DCS50) and the InnoZyme™ assay kit for human neutrophil elastase (Calbiochem, California, USA, CBA 016). These assays use a quantitative sandwich immunoassay technique. 100μL of assay diluent was added to each well followed by 100μL of standard or sample. The microplate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. Each well

was washed 4 times with a wash buffer. 200µL of either SDF-1, MMP-9 or G-CSF conjugate was added to each well and the microplate was again incubated for 2 hours at room temperature on the shaker. Washing was repeated and 200µL of substrate solution was added to each well whilst protected from the light. After incubation at room temperature for 30 minutes, stop solution was added to each well and the optical density measured using a microplate reader (Dynatech Laboratories, Chantilly, USA, MR5000). All determinations were performed in duplicate. Concentrations of SDF-1, MMP-9, G-CSF and NE were calculated by generating a standard curve, using the optical density obtained to determine the corresponding concentrations. Serum SDF-1 and G-CSF concentrations were expressed as pg/mL and MMP-9 and NE concentrations as ng/mL. The minimum detectable concentration levels for SDF-1, G-CSF, MMP-9 and NE was 18pg/mL, 0.4pg/mL, 0.156ng/mL and 0.063ng/mL.

### 5.2.3 Serum IL-8, IP-10, MIG, RANTES and MCP-1 chemokine analysis

Serum levels of IL-8, IP-10, MIG, RANTES and MCP-1 were quantified with the BD™ Cytometric Bead Array (CBA) kit (BD Biosciences, Cowley, UK, 552990), which uses a series of particles with discrete fluorescent intensities to simultaneously detect multiple soluble analytes. The BD CBA Capture Bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample. Three bead populations with distinct fluorescent intensities have been coated with monoclonal capture antibodies specific for IL-8, IP-10, MIG, RANTES and MCP-1.

For each sample, 25µl capture beads were mixed with 25µl PE-conjugated detection antibodies, and then incubated with 50µl of either recombinant chemokine standards or patient serum for 3 hours in the dark. Samples were washed in 1mL dilution media and then centrifuged at 200 x g for 5 minutes. Supernatant from each assay tube was aspirated and discarded and the bead pellet was resuspended with 300µl of dilution media. The samples were analysed in the FL3 channel of a FACS CALIBER® flow

cytometer and approximately 1800 events were gated in total. Sample results were generated in graphical and tabular formats using the BD™ CBA Analysis Software. Serum IL-8, IP-10, MIG, RANTES and MCP-1 concentration values were expressed as pg/mL. The minimum detectable concentration levels for IL-8, IP-10, MIG, RANTES and MCP-1 were 0.2pg/mL, 2.8pg/mL, 2.5pg/mL, 1.0 pg/mL and 2.7pg/mL respectively.

### 5.2.4 Patient details for SDF-1, MMP-9 and NE Immunohistochemistry

Archival liver biopsy tissue was used from 5 adult normal controls (NC) and compared with 4 patients who had a history of continuous alcohol consumption >400gms/week, no clinical or serological evidence of other causes of liver disease and had histological evidence of alcoholic hepatitis (AH) on biopsy. The following histological criteria were used for the diagnosis of AH in this study: 1) Macrovesicular and microvesicular steatosis, 2) polymorphonuclear cellular infiltrate, 3) intracellular accumulation of Mallory's hyaline/bodies and 4) evidence of hepatocellular injury or necrosis.

Ethical approval for the use of archival liver biopsy tissue in this study was granted by the Lothian Health Board Research and Ethics Committee (2003/R/GI/02). Sample sizes for the control and AH patient groups was limited in this study due to the restrictions placed by the ethical approval committee. Patient and clinical details for 3 out of the 5 control specimens labelled as normal were not available. These 3 NC samples labelled were re-examined by a pathologist (Prof DJ Harrison) and verified histologically that they appeared normal. The remaining 2 NC samples were from pre transplant female donor livers aged 47 and 62 years respectively, both died of subarachnoid haemorrhage and had no clinical history of pre-existing liver disease. Both tissue samples were documented as normal on histopathology reporting. The 4 AH liver biopsies specimens were from 1 female aged 52 and 3 male patients aged 38, 48, and 31 years respectively. Histopathological diagnosis of all liver samples were

based on the original pathological report and re-examined by a pathologist (Prof DJ Harrison).

### 5.2.5 Immunohistochemistry and cell counting for SDF-1, MMP-9 and NE

Immunohistochemical staining was performed on 3-micron-thick, formalin fixed paraffin embedded tissue sections using selected antibodies. After deparaffination and hydration, slides were microwaved on high power for 20 minutes in 2.1gm/L Citrate (pH6) solution and then blocked in 1% $H_2O_2$ for 15 minutes. Sections were incubated with a single selected primary antibody: Neutrophil elastase mouse mAb (DakoCytomation, M0752) 1:100 dilution at room temperature, SDF-1 rabbit anti-human mAb (Pasteur Institute, Paris, France, K15C) 1:50 dilution at 4°C overnight and MMP-9 goat anti-human mAb (Biogenesis, Poole, UK, 5980-0911) 1:100 dilution at 4°C overnight. The NE and SDF-1 labelled slides were washed and then incubated for 40 minutes at room temperature with an HRP polymer-conjugated detection antibody (DakoCytomation EnVision™ + System-HRP, K4006/K4010). The MMP-9 labelled slides were washed and incubated for 30 minutes in a 1:400 dilution of biotinylated rabbit anti-goat immunoglobulins (DakoCytomation, E0466) followed by wash and a further 30 minutes incubation with an Avidin-Biotin Horseradish Peroxidase reagent (Vectastain Elite ABC kit, PK6100). All slides were then washed and incubated with DAB substrate chromagen (DakoCytomation) and counterstained with Mayer's haematoxylin.

For SDF-1 staining, each section was screened on low power (200x) under a light microscope (Olympus B061, Japan) and then a minimum of 5 portal tracts from each biopsy specimen was photographed at a magnification of 320x using a colour digital camera (Pixera Penguin Pro 150ES, USA). Two separate assessors who were blinded to the nature of the biopsies viewed all sections from the digital images taken at 320x magnification and determined the SDF-1 staining intensity per sample. The intensity

of SDF-1 staining was graded semi-quantitatively from 0-3+ and a median score was taken for the NC and AH group. The scale was defined as follows: 0= negative, 1+ = weakly positive, 2+ = moderately positive, 3+ = strongly positive.

To evaluate the amount of positive nuclei staining for MMP-9 and NE, each section was visualised under a light microscope (Olympus B061, Japan) and 10 random fields per liver biopsy were selected and photographed at 320x magnification using a colour digital camera (Pixera Penguin Pro 150ES, USA). Two separate assessors who were blinded to the nature of the biopsies viewed all sections from the digital images taken at 320x magnification and counted the amount of positive staining nuclei per field.

### 5.2.6 Statistics

All statistical analysis was performed on SPSS® 12.0 for Windows® software (SPSS Inc. Chicago, IL). Results are expressed as the Mean with Standard Error of the Mean (SEM) for individual group data. Differences between groups in the serum chemokine study were analysed with a one way analysis of variance (ANOVA) test with post-hoc Dunnets test to compare each group with NC. For the immunohistochemistry studies the Mann-Whitney U statistical test (non-parametric significance) was used to determine whether the differences between the AH and NC groups were significant. Results were considered significant when $p<0.05$. The Cohen's kappa coefficient was used to determine inter-observer agreement on staining intensity and cell counts in the liver sections.

## 5.3 Results

### 5.3.1 Serum SDF-1, MMP-9 and G-CSF levels are significantly elevated in patients with alcoholic hepatitis

Patients with AH had significantly increased serum SDF-1 and MMP-9 levels (1440 ± 300 pg/mL, $p<0.05$; 322.9 ± 86.40 ng/mL, $p<0.05$) as compared with the NC group (393 ± 92 pg/mL, 85.29 ± 26.47 ng/mL). There was no significant increase in serum SDF-1 or MMP-9 levels in either the acute paracetamol (977 ± 135 pg/mL, $p>0.05$; 69.94 ± 10.82 ng/mL, $p>0.10$) or chronic liver injury groups (1071 ± 112 pg/mL, $p>0.05$; 133.88 ng/mL, $p>0.10$) when compared with the NC group ***(see figure 25 - 26)***. Serum G-CSF levels in the AH group was significantly increased (34.23 ± 10.19 pg/mL, $p<0.01$) as compared with the NC group (1.03 ± 1.03 pg/mL). There was no significant increase in serum G-CSF levels in either the acute paracetamol (6.13 ± 5.79 pg/mL, $p>0.05$) or chronic liver injury groups (6.29 ± 1.96 pg/mL, $p>0.05$) as compared with NC ***(see figure 27)***.

The serum NE levels were significantly elevated in only the AH group (1.81 ± 0.45 ng/mL, $p<0.05$) when compared to NC (0.33 ± 0.12 ng/mL) ***(see figure 28)*** but this rise in NE did not correlate with the corresponding serum MMP-9 rise ($r^2= 0.0427$) nor with the circulating $CD34^+$ levels ($r^2= 0.1883$) in AH patients. This observed rise in NE levels is most likely due to activation of peripheral blood neutrophils as AH liver injury is a neutrophil driven process and is characterised by a peripheral blood neutrophilia. Our AH patient group also demonstrated a significant rise in circulating neutrophil count (10.6 ± 2.07 x $10^9$/l, $p<0.05$) as compared with the NC (3.32 ± 0.46 x $10^9$/l). There was no significant difference in serum NE levels in either the acute paracetamol (0.10 ± 0.02 ng/mL, $p>0.10$) or chronic liver injury groups (0.83 ± 0.25ng/mL, $p>0.05$) as compared with NC. Likewise, there was no significant increase in neutrophil counts in either the acute paracetamol (4.41 ± 0.72 x $10^9$/l) or chronic liver injury group (4.81

$\pm$ 0.78 x $10^9$/l) suggesting there was not significant neutrophil activation in these forms of liver injury.

### 5.3.2 Serum IL-8, IP-10, MIG, RANTES and MCP-1 levels demonstrate a non specific response to liver injury

Serum IL-8 levels were significantly raised in all types of liver injury (AH: 149.14 $\pm$ 44.18 pg/mL, $p<0.001$; acute paracetamol: 113.06 $\pm$ 88.50 pg/mL, $p<0.01$; chronic injury: 33.23 $\pm$ 7.54 pg/mL, $p<0.01$) when compared with NC (3.93 $\pm$ 0.65pg/mL) ***(see figure 29)***. Furthermore there was no correlation between IL-8 levels and MMP-9 or $CD34^+$ levels. Serum IP-10 and MIG levels were significantly raised in the AH (570.42 $\pm$ 224.59 pg/mL, $p<0.05$ and 915.02 $\pm$ 485.48 pg/mL, $p<0.05$), acute paracetamol (757.06 $\pm$ 432.88 pg/mL, $p<0.05$ and 1796.5 $\pm$ 835.73 pg/mL, $p<0.01$) and chronic liver injury groups (516.37 $\pm$ 131.67 pg/mL, $p<0.05$ and 805.95 $\pm$ 201.59 pg/mL, $p<0.05$) when compared with NC (82.78 $\pm$ 15.41 pg/mL, $p<0.05$ and 116.23 $\pm$ 26.23 pg/mL) ***(see figure 30-31)***.

Serum RANTES levels were decreased in all the liver injury groups when compared to the NC (3593.27 $\pm$ 267.14 pg/mL, $p<0.05$), although this reduction was only statistically significant in the paracetamol (1611.98 $\pm$ 413.32 pg/mL, $p<0.05$) and chronic liver injury groups (1679.59 $\pm$ 182.70 pg/mL, $p<0.05$) and not to the AH group (2378.70 $\pm$ 586.97 pg/mL, $p>0.05$) ***(see figure 32)***. Serum MCP-1 levels were only significantly raised in the paracetamol injury group (441.18 $\pm$ 206.84 pg/mL, $p<0.05$) and not in the AH group (112.09 $\pm$ 13.74 pg/mL, $p>0.10$) or chronic liver injury group (127.24 $\pm$ 24.27 pg/mL, $p>0.10$) when compared to NC (77.69 $\pm$ 15.83 pg/mL) ***(see figure 33)***.

### 5.3.3 SDF-1 expression is reduced and MMP-9/NE expression unaltered in the livers of patients with alcoholic hepatitis

Examination of the liver tissue sections demonstrated positive SDF-1 staining on biliary epithelial cells in both the NC and AH group specimens. There was no staining for SDF-1 in the hepatocytes, inflammatory cells, vascular epithelial cells or other liver parenchymal cell components in either the AH or NC sections. Overall there was less intense SDF-1 staining of the biliary epithelium in the AH sections as compared with the NC group (median staining intensity 1+ *vs.* 2+ (range 1-3). ***(see figures 34-35 and table 8).*** The Cohen's kappa coefficient for the inter-observer agreement on SDF-1 staining intensity was 0.7714.

In both the NC and AH sections MMP-9 staining was noted predominately in neutrophils within the hepatic parenchyma as well as in activated Kupffer cells of the hepatic sinusoids. No MMP-9 staining was observed within hepatocytes or biliary epithelium in either the NC or AH group. Staining for MMP-9 in all liver sections was uniform and did not vary in intensity. There was no apparent difference in the amount of MMP-9 positive staining cells between the AH and NC groups ($7.37 \pm 1.17$ cells per field *vs.* $7.32 \pm 1.43$ cells per field, $p>0.10$) ***(see figures 36-37)***. The Cohen's kappa coefficient for the inter-observer agreement on MMP-9 cell counting was 0.6909.

Positive NE staining was identified in neutrophils within the hepatic parenchyma as well as in the hepatic sinusoids in both the NC and AH sections. No NE staining was observed within hepatocytes or biliary epithelium in either group. In all liver sections staining for NE was uniform and did not vary in intensity. There was no difference in the amount of NE positive staining cells between the AH and NC groups ($9.05 \pm 3.14$ cells per field *vs.* $8.45 \pm 1.71$ cells per field, $p>0.10$) ***(see figures 38-39)***. The Cohen's kappa coefficient for the inter-observer agreement on NE cell counting was 0.6701.

## 5.4 Conclusion

We demonstrated that AH patients (whose circulating $CD34^+$ counts are elevated) have significantly increased serum SDF-1, MMP-9 and G-CSF levels as compared to controls in non time course blood samples. We failed to demonstrate a similar increase in SDF-1, MMP-9 and G-CSF levels in other forms of acute and chronic liver injury studied. Serum NE, IL-8, IP-10, MIG, RANTES and MCP-1 levels demonstrated non specific responses to alcohol induced liver injury. Patients with severe AH were treated with Pentoxifylline which may have had an influence on the chemokine profile results. Hepatic expression of SDF-1 was reduced in AH whilst MMP-9 and NE expression was unaltered in the livers of AH patients when compared to controls.

**Table 7**

| | **Alcoholic Hepatitis** | **Acute Paracetamol Injury** | **Chronic Liver Injury of variable aetiology** | **Normal Controls** |
|---|---|---|---|---|
| Mean Age in years | 49.2 ± 3.8 | 36.5 ± 4.2 | 50 ± 2.1 | 28.3 ± 3.1 |
| Male: Female Ratio | 5:6 | 4:1 | 11:9 | 6:2 |
| Group Numbers | 11 | 5 | 20<br>(HCV =5)<br>(PBC =6)<br>(AAC =9) | 8 |
| Serum bilirubin concentration (μmol/l)* | 313.4 ± 78 | 128 ± 27 | 46.9 ± 12 | 25 ± 5.9 |
| Serum ALT concentration (IU/l)* | 54.9 ± 11.8 | 4337 ± 421 | 50.1 ± 8.1 | 28 ± 5.3 |
| Serum albumin concentration (g/l)* | 29.1 ± 1 | 28.4 ± 1.2 | 37 ± 1.8 | 47 ± 2.1 |
| Prothromin Time (seconds)* | 25.8 ± 10.5 | 36.8 ± 5.8 | 11.93 ± 0.68 | N/A |

**Table 7: Patient characteristics and biochemical parameters of serum chemokine sample groups.**

*Data represents mean values for each group ± SEM. AAC: abstinent alcoholic cirrhosis; PBC: primary biliary cirrhosis; HCV: hepatitis C virus; ALT: alanine-amino transferase; N/A: not available.

**Table 8**

| Liver biopsy | Median SDF-1 staining intensity (range) |
|---|---|
| Normal (n=5) | 2+ (1-3) |
| AH (n=5) | 1+ (1-3) |

**Table 8: Staining intensity of SDF-1 reduced in AH liver sections when compared to NC.**

N values represents the number of samples in each group.

**Figure 25**

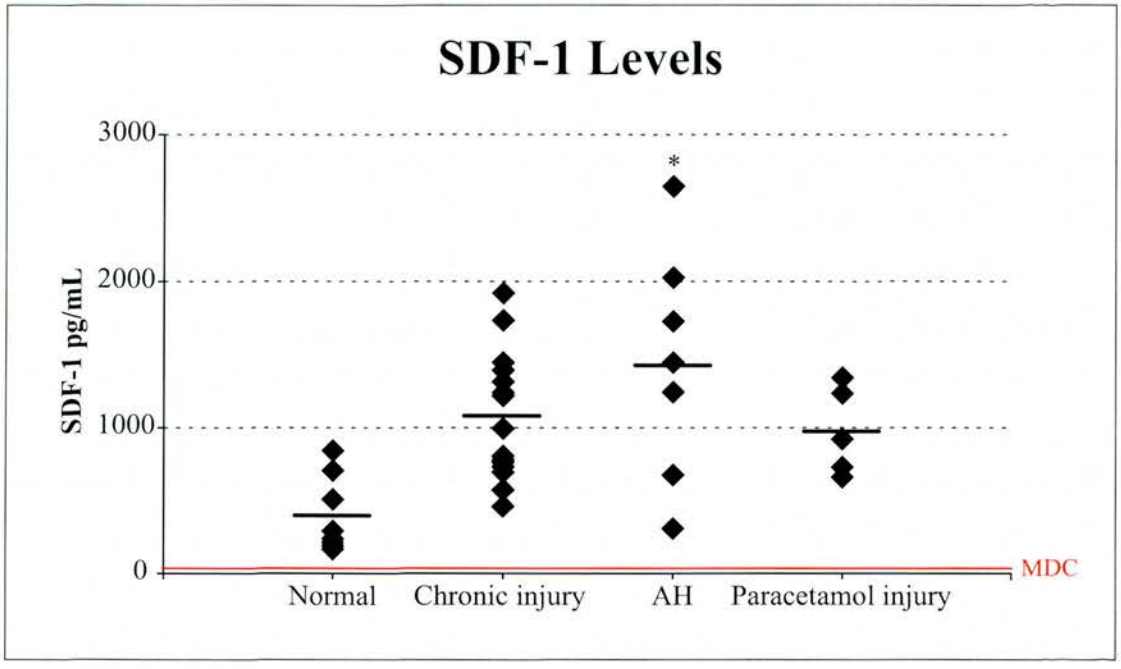


**Figure 25: Serum SDF-1 levels are significantly increased in AH patients compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration. *$p<0.05$ compared to normal control.

**Figure 26**

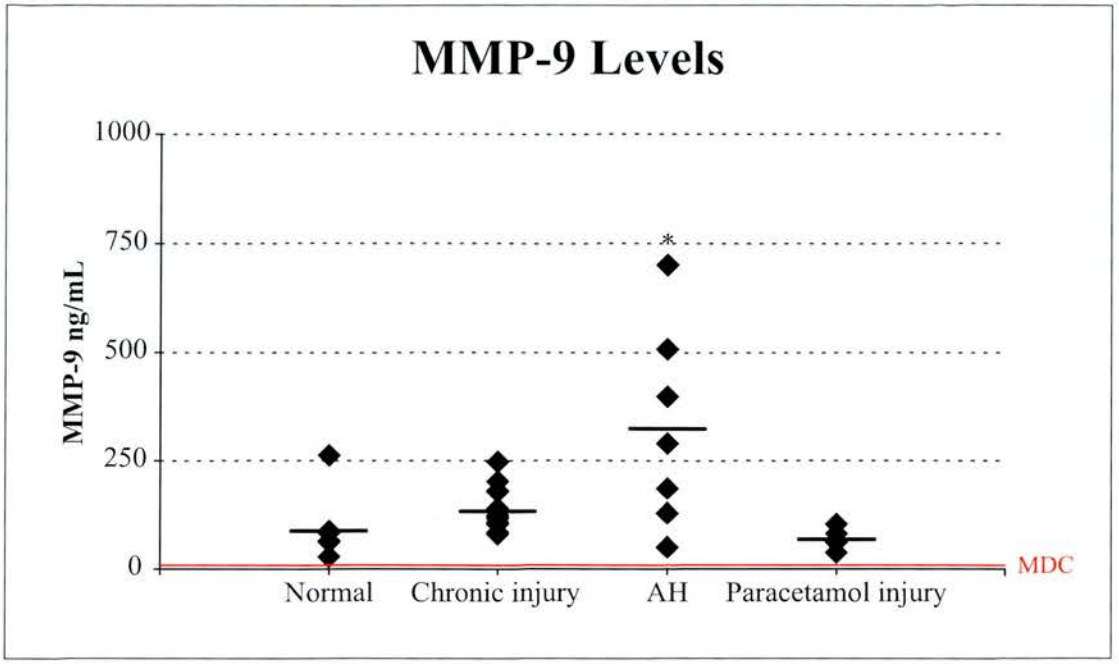


**Figure 26: Serum MMP-9 levels are significantly increased in AH patients compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration.*$p<0.05$ compared to normal control.

**Figure 27**

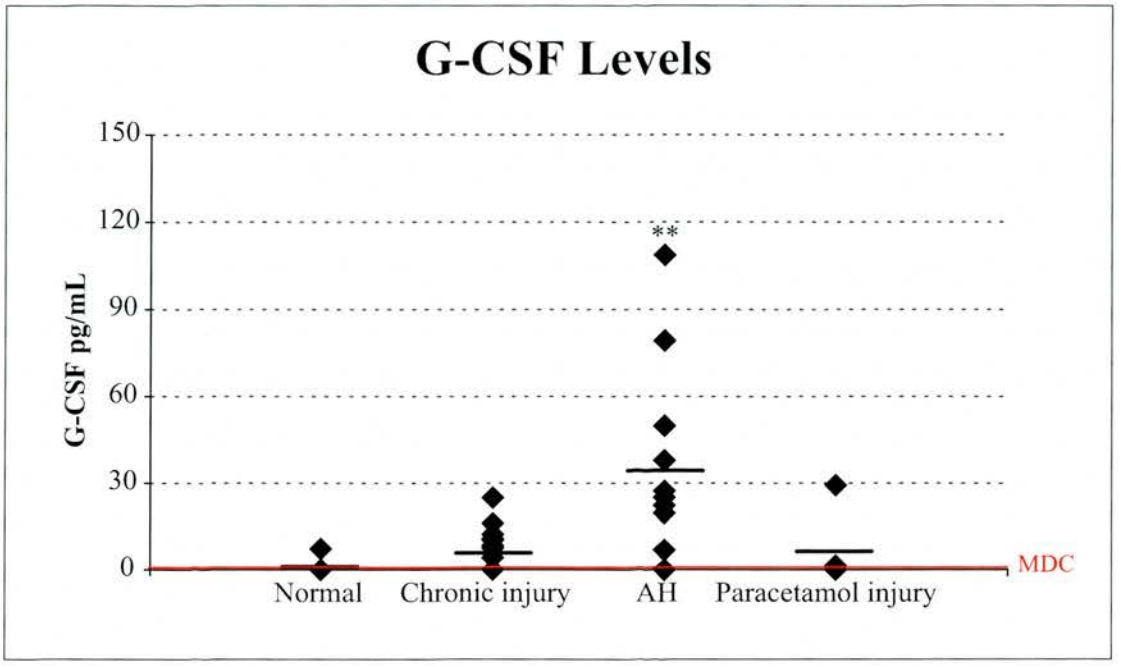


**Figure 27: Serum G-CSF levels are significantly increased in AH patients compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration. **p<0.01 compared to normal control.

**Figure 28**

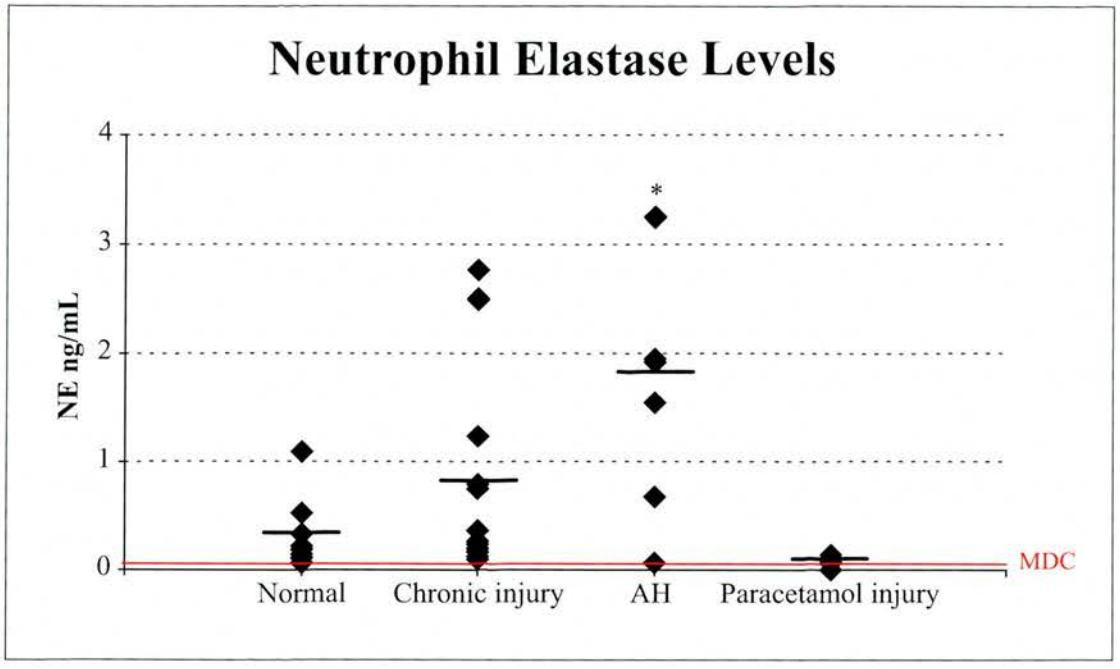


**Figure 28: Serum NE levels are significantly increased in AH patients compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration. *$p<0.05$ compared to normal control.

**Figure 29**

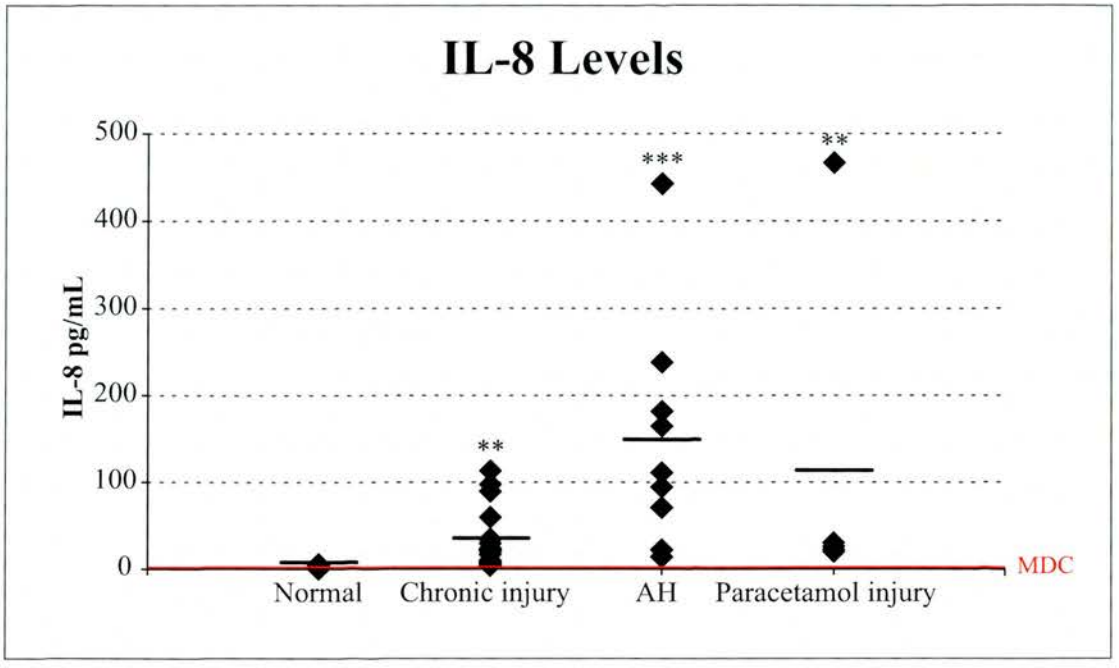


**Figure 29: Serum IL-8 levels are significantly increased in all liver injury groups compared to NC.**
Individual and mean values are represented for each group. MDC: Minimal detectable concentration. **p<0.01 compared to normal control. ***p<0.001 compared to normal control.

**Figure 30**

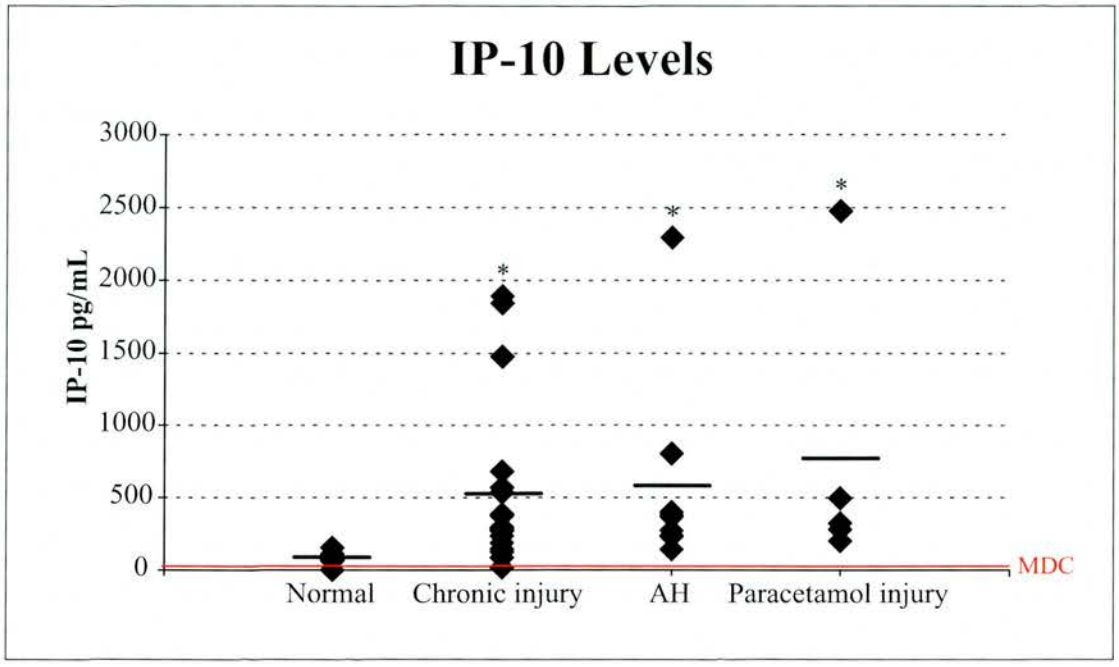


**Figure 30: Serum IP-10 levels are significantly increased in all liver injury groups compared to NC.**
Individual and mean values are represented for each group. MDC: Minimal detectable concentration. *$p<0.05$ compared to normal control.

**Figure 31**

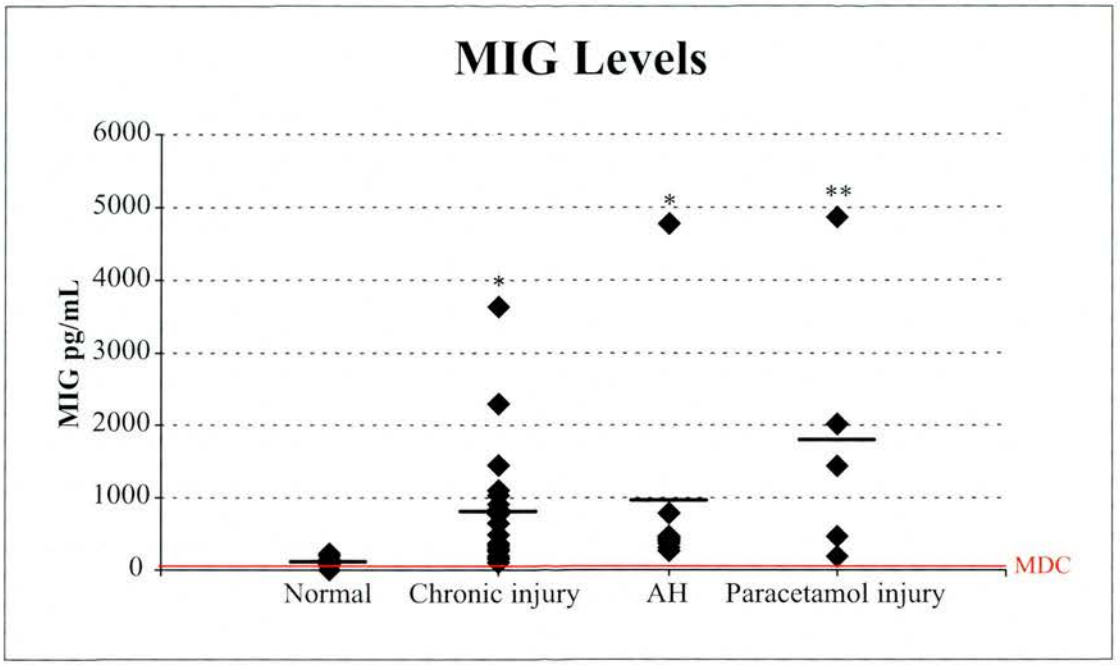


**Figure 31: Serum MIG levels are significantly increased in all liver injury groups compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration. *$p<0.05$ compared to normal control. **$p<0.01$ compared to normal control.

**Figure 32**

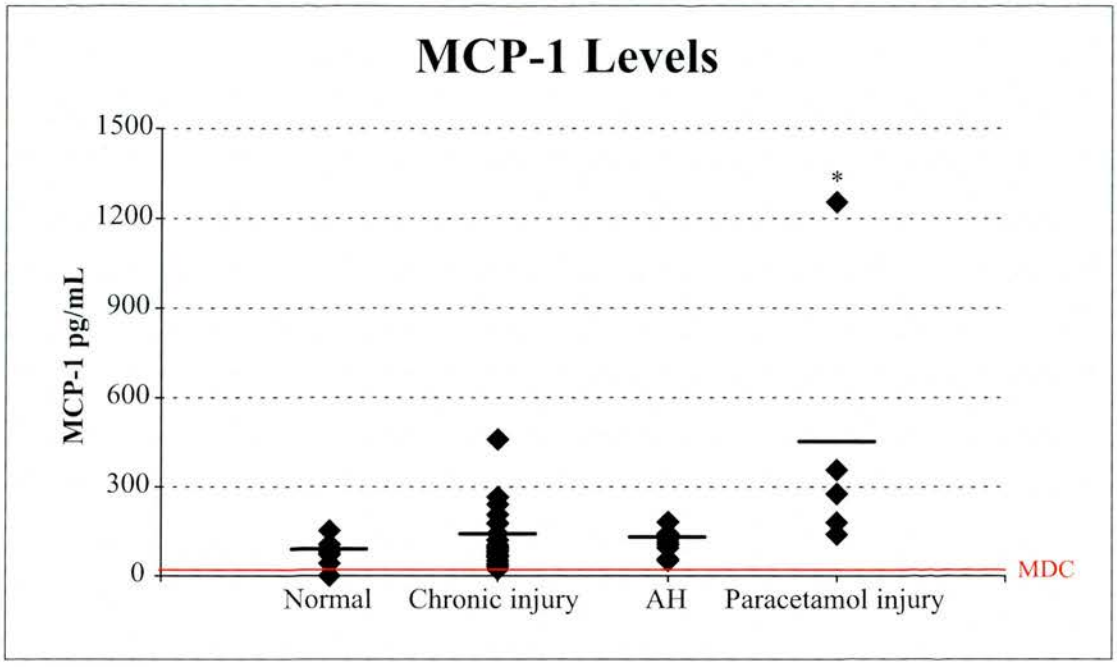


**Figure 32: Serum RANTES levels are not significantly altered in AH patients compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration. *$p<0.05$ compared to normal control.

**Figure 33**

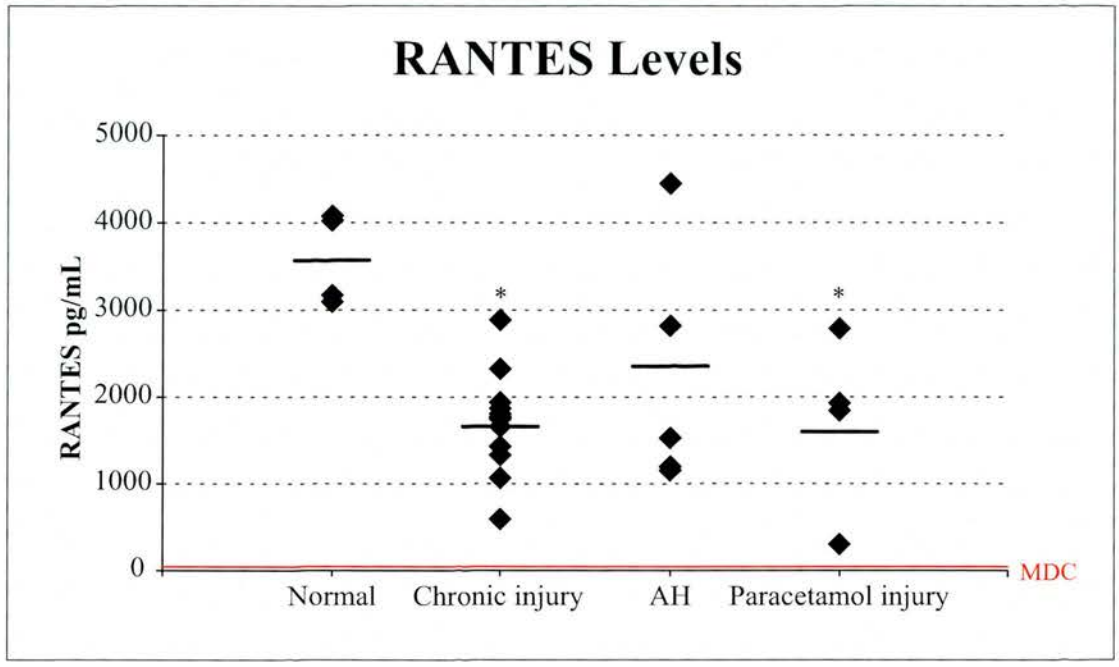


**Figure 33: Serum MCP-1 levels are not significantly altered in AH patients compared to NC.**

Individual and mean values are represented for each group. MDC: Minimal detectable concentration. *$p<0.05$ compared to normal control.

**Figure 34**

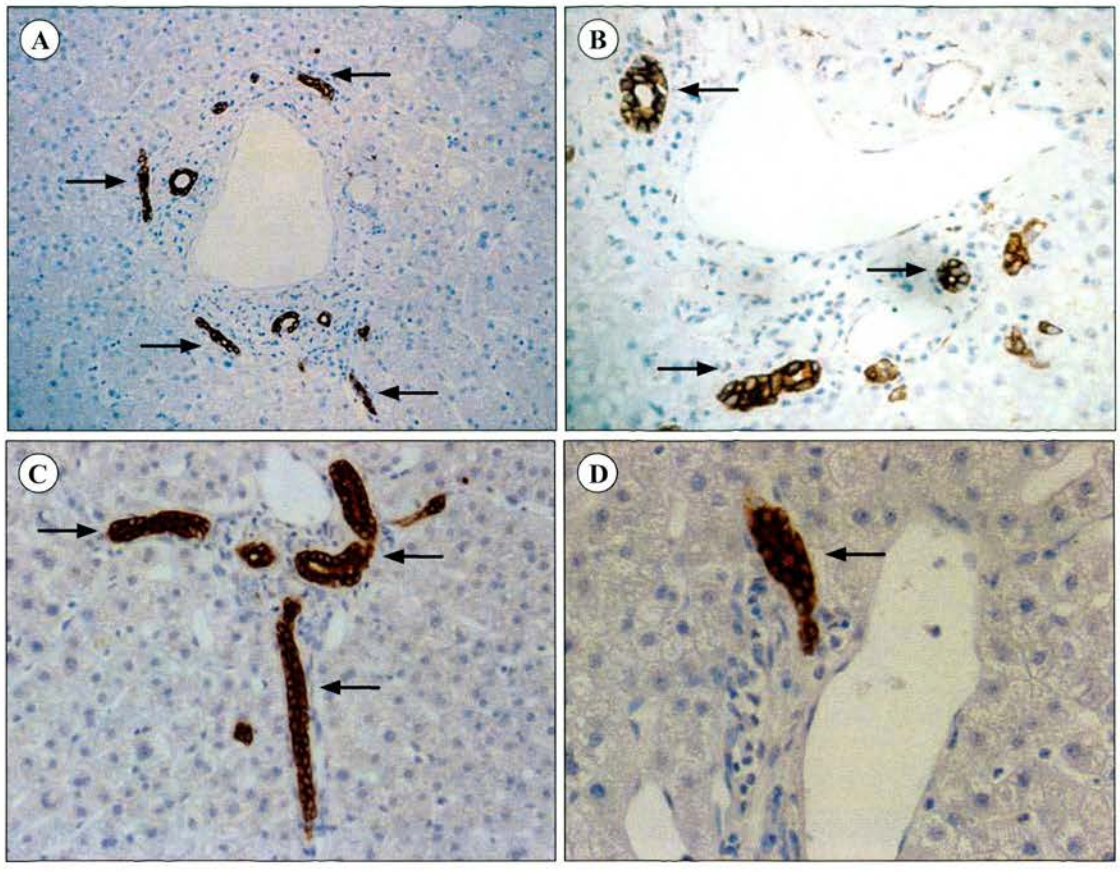


**Figure 34: Immunohistochemical staining for SDF-1 in normal liver sections.**

**A-D)** Liver biopsy sections from normal controls stained for SDF-1 *(brown)*. Intense SDF-1 staining is only expressed in the biliary epithelium *(arrows)*. Original magnification: A,100x; B-C, 200x; D, 320x.

**Figure 35**

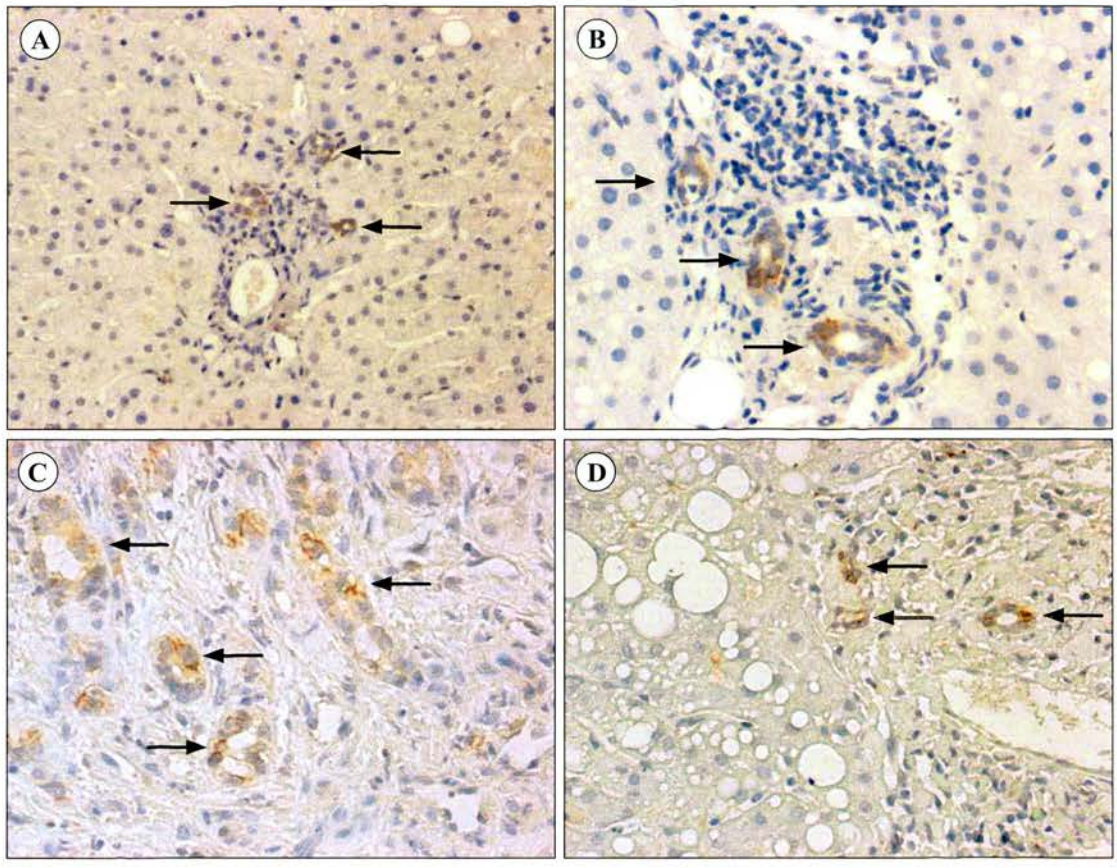


**Figure 35: Immunohistochemical staining for SDF-1 in AH liver sections.**

**A-D)** Liver biopsy sections from patients with AH stained for SDF-1 *(brown)*. Less intense SDF-1 staining is visualised in the biliary epithelium *(arrows)*. Original magnification: A, 200x; B-D, 320x.

Figure 36

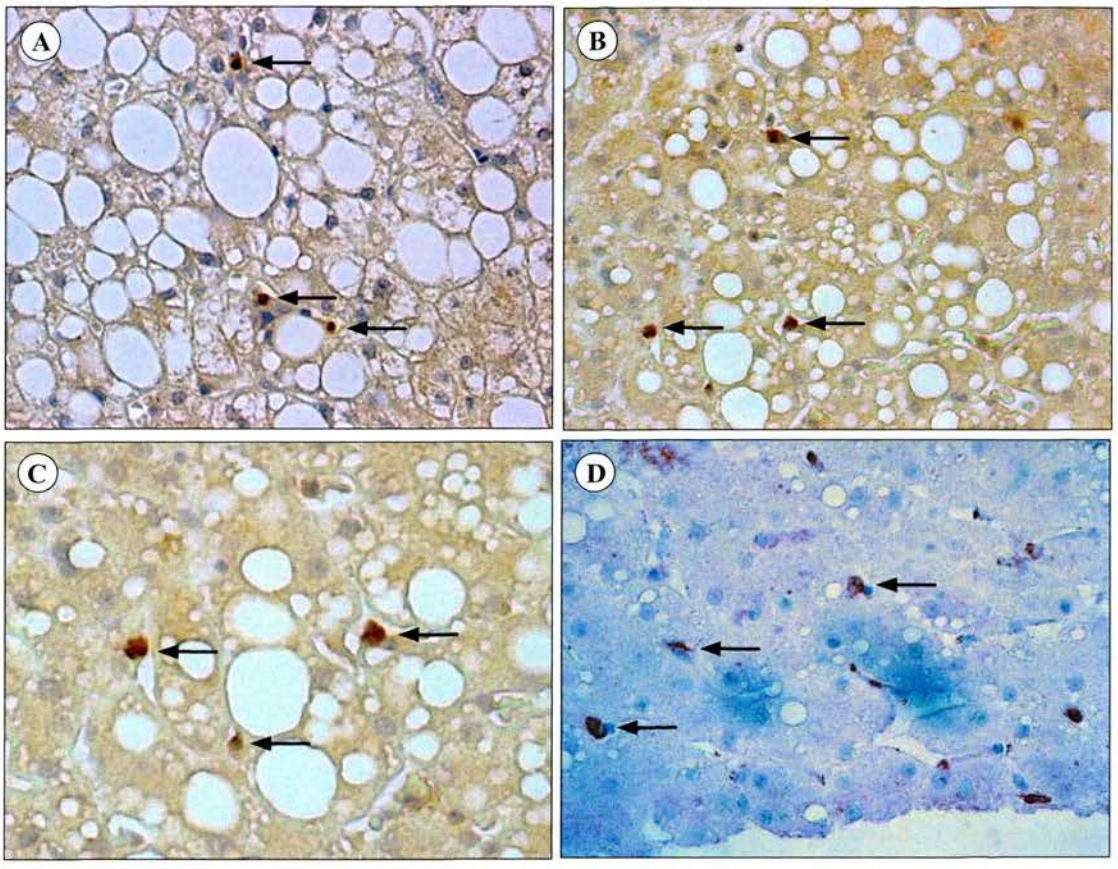


**Figure 36: Immunohistochemical staining for MMP-9 in AH liver sections.**

**A-D)** Liver biopsy sections from patients with AH stained for MMP-9 *(brown)*. MMP-9 staining was noted in neutrophils within the hepatic parenchyma as well as in activated Kupffer cells *(arrows)*. Original magnification: A-D, 320x. Images C & D have been magnified to demonstrate neutrophil MMP-9 staining.

**Figure 37**

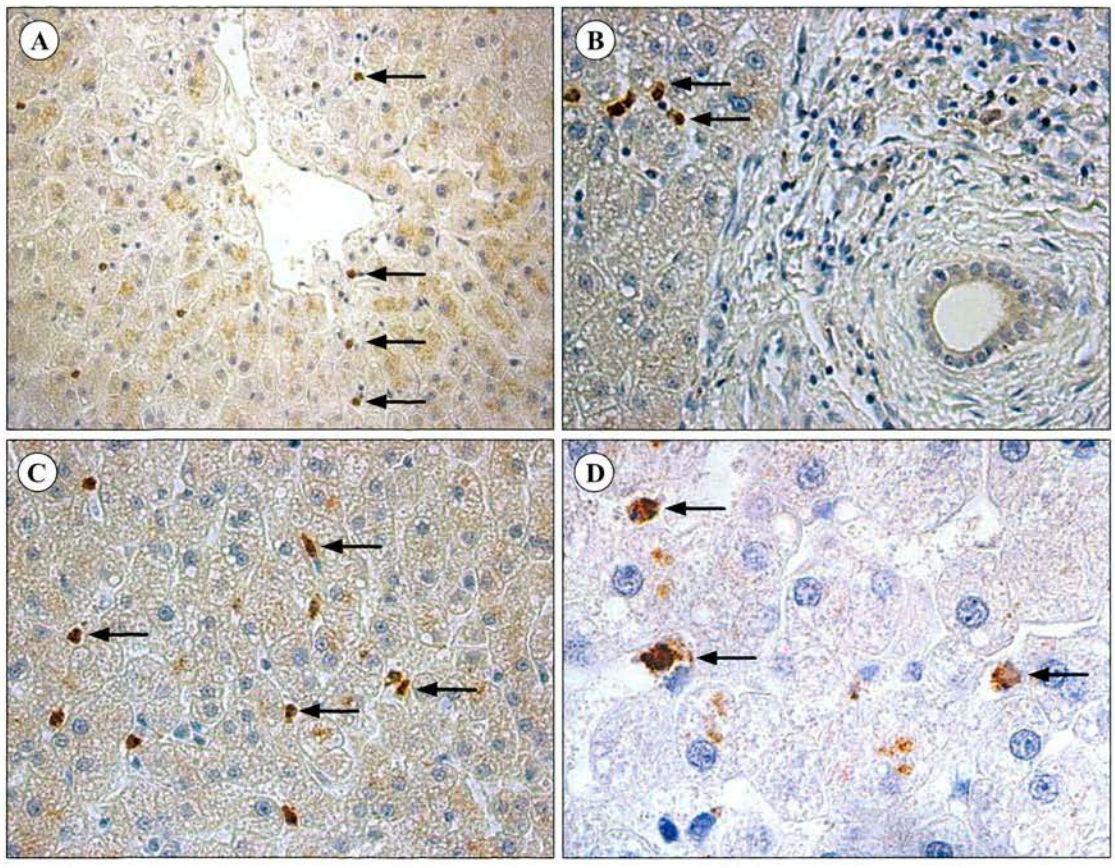


**Figure 37: Immunohistochemical staining for MMP-9 in normal liver sections.**

**A-D)** Liver biopsy sections from normal controls stained for MMP-9 *(brown)*. MMP-9 staining was noted predominately in neutrophils within the hepatic parenchyma as well as in activated Kupffer cells of the hepatic sinusoids *(arrows)*. Original magnification: A, 200x; B-C, 320x; D, 600x.

**Figure 38**

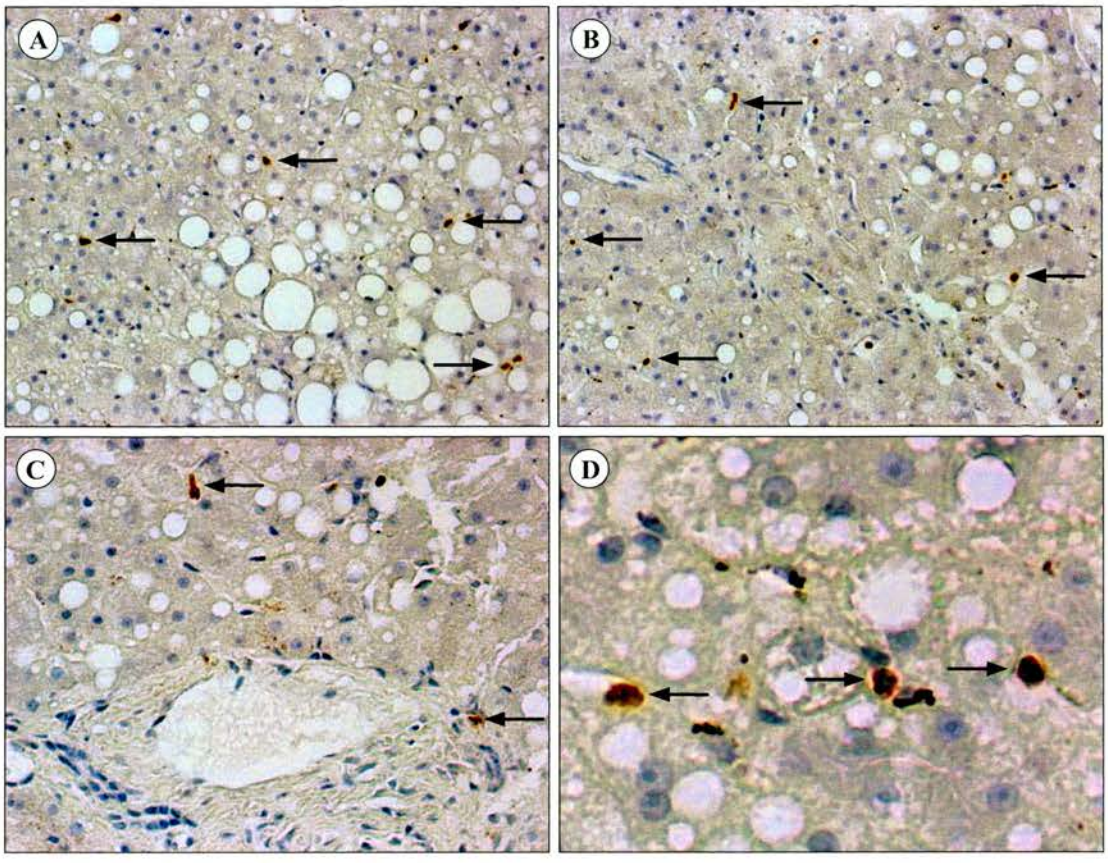


**Figure 38: Immunohistochemical staining for neutrophil elastase (NE) in AH liver sections.**

**A-D)** Liver biopsy sections from patients with AH stained for NE *(brown)*. NE staining was noted in neutrophils within the hepatic parenchyma *(arrows)*. Original magnification: A-B, 200x; C, 320x; D, 600x.

**Figure 39**

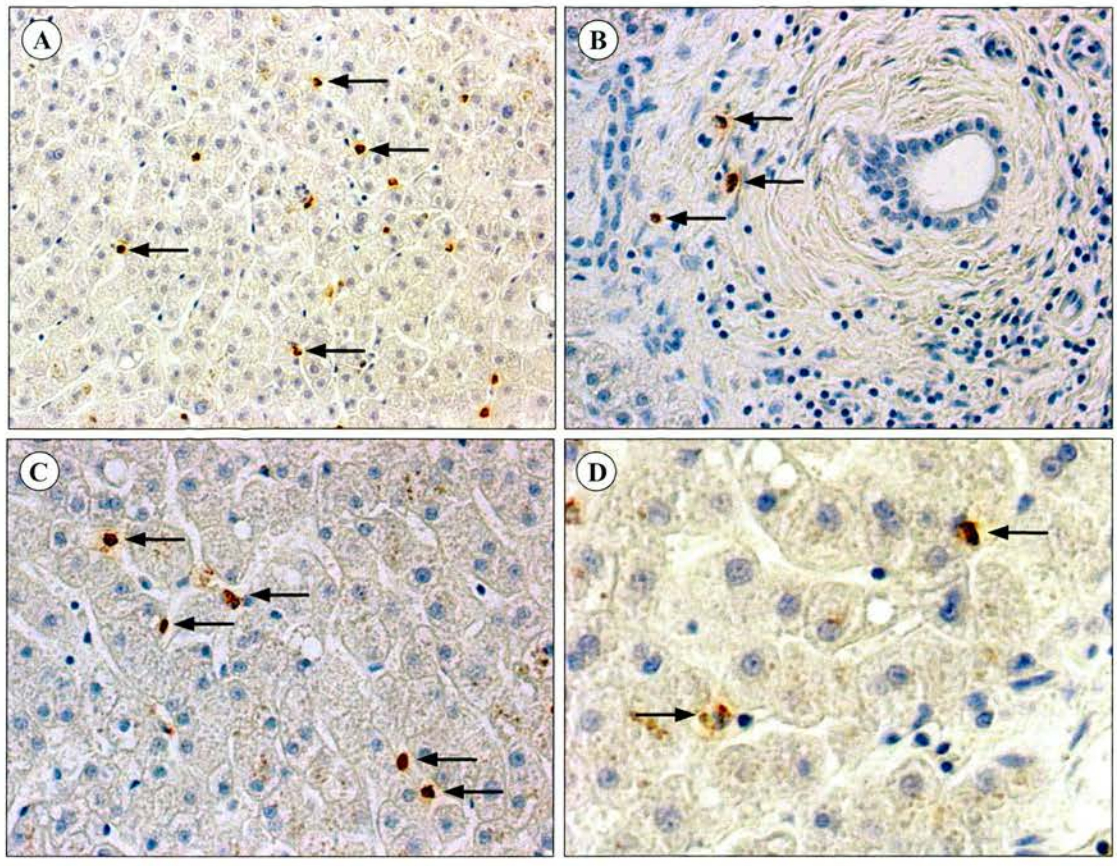


**Figure 39: Immunohistochemical staining for neutrophil elastase (NE) in normal liver sections.**

**A-D)** Liver biopsy sections from normal controls stained for NE *(brown)*. NE staining was noted in neutrophils within the hepatic parenchyma *(arrows)*. Original magnification: A, 200x; B-C, 320x; D, 600x.

# CHAPTER 6

# Discussion

In this thesis we attempt to address an important area of research in both basic and clinical hepatology: The role of BM-derived stem/progenitor cells in liver fibrosis and regeneration. We aimed to study this in clinical patients and focus on a subset of patients with alcohol related liver injury in which the contribution of the bone marrow has not been extensively studied. The studies presented in this thesis were designed to answer the following specific questions:

1. Does mobilisation and hepatic recruitment of HSCs occur in alcohol induced liver injury and if such mobilisation contributed to a reparative process?

2. Do mobilised HSC in alcohol induced liver injury display true stem cell potential properties?

3. Do inflammatory cytokines and chemokine axes regulate the mobilisation and hepatic recruitment of HSCs in alcohol induced liver injury?

## 6.1 Mobilisation of circulating HSCs in alcohol induced liver injury

We have demonstrated that $CD34^+$ HSCs levels from non time course blood samples were elevated in patients with acute alcoholic hepatitis, suggesting that there was mobilisation of $CD34^+$ cells into the circulation. We failed to demonstrate a similar significant elevation in circulating $CD34^+$ counts in other acute or chronic liver injury groups studied, suggesting that this mobilisation of $CD34^+$ HSCs may be specific for AH and not a general response to liver injury. The observed elevated $CD34^+$ numbers could have arisen through a failure of $CD34^+$ cells to engraft, both back into the bone marrow or into the liver and thus create a slow increase in the number of circulating $CD34^+$ cells. However, it is likely that outside of the bone marrow niche environment, HSCs would be unable to remain undifferentiated. HSC's differentiate into myeloid, lymphoid and mesenchymal stem cells and give rise to populations of macrophages, lymphocytes and myofibroblasts. Most of these cells would retain $CD34^+$ expression, but lose it shortly after lineage specific differentiation. Hence, circulation of $CD34^+$ cells is likely to be short lived, and a slow build up of these cells would be unlikely.

We recognise that a single blood sample specimen provides only one snapshot interpretation and may not be the most appropriate time point temporally. Mobilisation of $CD34^+$ cells during liver injury may also occur in waves, and our results may be influenced by the timing of our samples in relation to the kinetics of the process. This issue could be addressed by collecting serial blood samples on sequential days from patients (Days 1, 3, 5, 7, 10, and 14) and analysing the $CD34^+$ cell count for the different time points. Samples would be taken not only from the AH patients but also from the normal controls, acute paracetamol injury patients and chronic liver injury patients to demonstrate any temporal variation in $CD34^+$ levels and to help us understand the kinetics of $CD34^+$ mobilisation in AH and other forms of liver injury.

The patient sample groups included in the peripheral blood $CD34^+$ study were selected to be representative of a broad range of acute and chronic liver injury as well as being sufficiently common to have clinical relevance and provide adequate numbers. In the AH group we aimed not to include patients with previous documented alcoholic cirrhosis, although this patient group is heterogeneous and there would be a proportion of patients with established alcoholic cirrhosis included even though it may not have been previously documented or identified. The chronic liver disease patients were all clinically stable and this group could be further optimised to include patients with more severe forms of these chronic diseases such as decompensated PBC and decompensated HCV cirrhosis. Inclusion of such groups could further clarify whether $CD34^+$ mobilisation was specific for AH or a more generalised response to severe forms of liver injury.

AH patients were screened to ensure that sepsis, bleeding, severe co-morbidities and prednisolone use would not be confounding factors that may have had an effect on the bone marrow and influence our results. However patients treated with Pentoxifylline for severe AH were included in this study as this was standard medical therapy for patients with severe AH and by excluding these patients from our study it would limit our sample size and be less representative of the spectrum of severity of AH patients. Pentoxifylline is not known to directly inhibit HSC mobilisation although it does modify the host immune responses and has an effect on cytokine expression such as IL-8, MCP-1 and RANTES that may have an effect on stem cell physiology. We recognise that Pentoxifylline could influence our results and one possible method of addressing this issue is by taking serial blood samples for $CD34^+$ cell counts from AH patients both pre and post Pentoxifylline treatment to see any temporal or quantitative effect it may have. These results could be compared with a time course evaluation of peripheral blood $CD34^+$ cell counts from AH patients not treated with Pentoxifylline and used as a reference point to demonstrate any variation in $CD34^+$ levels.

HSC mobilisation in AH has not been previously reported and studies on the extent of peripheral blood HSC mobilisation in acute or chronic liver injury is limited. A study by De Silvestro et al has demonstrated elevated peripheral blood HSC levels following extensive liver resection in patients with malignant, primary or secondary liver diseases (De Silvestro et al. 2004). The authors however did not investigate whether there were increased $CD34^+$ cell populations within the surgically operated liver and this raises the question of whether the phenomenon seen, was due to a non specific systemic response to surgical trauma or not. A more recent study by Lemoli et al demonstrated HSCs mobilisation to be more pronounced in liver transplant patients with ischaemic/ reperfusion injuries early after surgery (day 3+) as compared with patients undergoing liver resections suggesting that the greater the extent of liver tissue injury the higher the degree of HSC mobilisation (Lemoli et al. 2006).

In contrast, Di Campli et al failed to demonstrate a time course $CD34^+$ cell mobilisation in hepatectomy patients or in patients with acute decompensation of liver cirrhosis (Di Campli et al. 2005) and concluded that under these circumstances the liver can rely on mature hepatocytes or endogenous progenitor cells to regenerate and repair itself. Our data would support their conclusions because we failed to demonstrate mobilisation of $CD34^+$ cells in patients with acute paracetamol liver injury or established liver cirrhosis although these were not serial time course blood samples from these groups. It is possible that in these forms of liver injury, other modes of hepatic regeneration were sufficient and thus the contribution of HSCs from the peripheral circulation is not required (Sell 2001). It is possible that in specific forms of liver injury such as alcohol induced injury, where there is a specific inhibition of hepatocyte proliferation is there mobilisation and recruitment of circulating HSCs into the liver as a response to liver injury.

## 6.2 Stem cell potential of mobilised HSCs

The results from our colony forming assays demonstrate that the $CD34^+$ HSCs found in blood were functional and had stem cell properties, in that they could form colonies when incubated in the methylcellulose growth media. In addition, different types of colonies were produced from the assays proving that such cells were pluripotent. We were interested to find that there was an increased number of CFUs formed from sorted $CD34^+/CD45^+$ cells of AH patients as compared with NC group. This would indicate that mobilised HSCs from AH patients were not only as potent as NC but may also display a true stem cell potential at a level higher than that seen with HSCs from healthy controls. Although we did not perform CFU assays on circulating $CD34^+/CD45^+$ cells from acute paracetamol and other chronic liver injury patients, this could be undertaken in future studies and serve as a further control group to compare the effects of different types of liver injury on stem cell potential. The CFU assay has allowed us to demonstrate haematopoietic stem cell properties, although more relevant would be the ability to look at the potential of sorted $CD34^+/CD45^+$ cells to form hepatic cells or fibroblasts. Studies *in vitro* have been successful in differentiating bone marrow stem cells into hepatocyte marker expressing cells using bone marrow haematopoietic cells cultured with hepatocytes or with stem cell growth factor, hepatocyte growth factor, epidermal growth factor and fibroblast growth factor-4 (Okumoto et al.2003; Lemoli et al.2006). Liver specific gene and protein markers such as alpha feto protein, cytokeratin 8, cytokeratin 18, cytokeratin 19 and albumin can be detected with reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemical analysis. Likewise, bone marrow cells have been successfully cultured *in vitro* to produce fibroblast colony forming cells and expression of fibrocyte markers such as pro($\alpha$1)I collagen, fibronectin, and vimentin mRNA can be detected with RT-PCR (Castro-Malaspina et al. 1980;Ebihara et al. 2006).

Although we did not investigate for hepatic or fibroblast differentiation potential in mobilised $CD34^+/CD45^+$ cells in our study, these methods could be adapted for use in future studies to evaluate whether these circulating cells have increased hepatic or fibroblast differentiation potential.

Our observation that $CD34^-/CD45^+$ cells were also producing higher total CFU counts in the AH group as compared with NC would suggest that other non $CD34^-$ progenitor cells (with pluripotent stem cell properties) such as MSC, multipotent adult progenitor cells (MAPC), $CD133^+/ CD34^-$ endothelial progenitor cells and side population (SP) cells may also be released into the peripheral circulation in alcohol induced liver injury and would merit further study.

## 6.3 HSCs hepatic recruitment and contribution to liver fibrosis

### 6.3.1 Bone marrow derived $CD34^+/CD31^-$ cells and hepatic progenitor cells

The liver biopsies from cross-sex liver transplant patients with acute alcohol injury demonstrated increased $Y^+$ recipient $CD34^+/CD31^-$ cells whilst the liver biopsies from non transplant AH patients contained a greater number of $CD34^+/CD31^-$ and $c\text{-}kit^+$/mast $cell^-$ stem cells when compared with controls. The co-localisation of Y-chromosome and $CD34^+/CD31^-$ markers in the alcohol injured cross-sex liver biopsies would suggest that these cells were of bone marrow haematopoietic stem cell origin although it has been documented that fibrocytes in human peripheral blood can also express the haematopoietic cell marker $CD34^+$ and the leukocyte marker $CD45^+$ (Bucala et al. 1994). Other markers for HSCs such as $c\text{-}Kit^+$, $Thy^+$, $Lin^+$, $Sca\text{-}1^+$ and $IL\text{-}7R^+$ have been described and these markers could be used in FISH studies to better characterise the bone marrow lineage of $Y^+$ recipient $CD34^+/CD31^-$ cells. We have demonstrated that non transplant AH liver biopsies contained a greater number of $CD34^+/CD31^-$ and $c\text{-}Kit^+$/$mast\ cell^-$ hepatic progenitor cells stem cells. There are however other markers

for hepatic stem cells such as epithelial cell adhesion molecule (EpCAM), neural cell adhesion molecule (NCAM) and cytokeratin 19 and these markers could be used in future work to further characterise the hepatic stem cells content in alcohol induced liver injury biopsies (Zhang et al. 2008).

We acknowledge that the numbers of patients studied to assess the contribution of bone marrow stem cells to hepatic parenchyma, myofibroblasts and progenitor populations was small and a greater numbers of patients would further bolster the validity of these observations. This could be achieved by obtaining ethical approval to extend access to archival liver transplant biopsies tissue taken at the Royal Infirmary of Edinburgh from 1993 up to present day, as well as accessing cross sex transplant biopsies tissue from other transplant units. The cross sex liver biopsy specimens from alcohol injury patients provide only one snapshot interpretation of a dynamic process and the timing of the biopsies was highly variable (13-63 months). By obtaining greater number of alcohol injured cross-sex transplant biopsy specimens we could analyse whether duration of bone marrow stem cell engraftment and contribution to the myofibroblast/ parenchymal population is altered over time. Furthermore an additional control group could be included to look at cross sex transplant biopsies from patients with no significant evidence of liver injury but at similar time points to those of the alcohol injury group. The normal control group in the hepatic progenitor study was sub-optimal in that clinical data was not available for some samples. The limited control sample size could however be expanded to include more pre transplant donor biopsies in which clinical and biochemical data would be available including liver biopsies from living related liver donors.

### 6.3.2 Bone marrow derived myofibroblasts

We were interested to observe that there was little contribution of BM derived stem cells to hepatocyte or biliary cell lineages. Instead, we observed that BM derived recipient ($Y^+$) cells contributed to the myofibroblast population ($\alpha$-$SMA^+$) within

the alcohol injured liver of transplant patients. We did not specifically address the mechanisms by which $Y^+$/ $\alpha$-$SMA^+$ cells contributed to the myofibroblast population and this could have occurred either via spontaneous cell fusion of recipient cells with activated hepatic stellate cells or myofibroblasts in the transplanted liver, or BM recipient cells acquiring a myofibroblast phenotype through trans-differentiation. One possible way of addressing the above scenario would be co-staining for Y-chromosome and X-chromosome using FISH in the cross-sex liver transplant biopsies. In the case of fusion we would expect to see XXXY cells, but in the case of trans-differentiation we would expect to see XY or XYXY cells. Considering the rodent study by Kisseleva et al which demonstrated that BM derived cells contributed via transdifferentiation to collagen producing fibrocytes then it would seem more probable that a similar process of myofibroblast transdifferentiation would occur in transplant patients with alcohol induced liver injury (Kisseleva et al. 2006). We did not further characterise the $Y^+$/ $\alpha$-$SMA^+$ myofibroblast cells for other fibrocyte markers such as vimentin or fibulin-2 nor test for functionality of the $Y^+$/ $\alpha$-$SMA^+$ cells although further proof of their fibrogenic potential could be evaluated using a method described by Forbes et al whereby active collagen transcription in myofibroblasts is demonstrated by in situ hybridisation for pro ($\alpha$1) I collagen expression (Forbes et al. 2006).

### 6.3.3 $CD34^+$ hepatic myofibroblasts

A proportion of the myofibroblasts co-stained for CD34 suggesting that a population of $CD34^+$ cells within the liver was contributing to the hepatic myofibroblast population. We have been unable to categorally demonstrate whether these $CD34^+$/$\alpha$-$SMA^+$ cell populations were of bone marrow haematopoietic stem cell origin or not due to technical difficulties of simultaneous staining for CD34, CD31, $\alpha$-SMA and Y-chromosome on the same tissue section. One possible method of addressing this challenge is to use serial 3 micron thick sections and stain neighbouring sections for CD34/ CD31/Y and CD34/$\alpha$-SMA/Y respectively as this procedure would allow for matching fields to be

as close as possible to each other. We did however demonstrate that a number of true progenitor cells ($CD34^+/CD31^-$) and $\alpha$-$SMA^+$ cells were $Y^+$ chromosome, as well as a number of these $CD34^+$ cells co-expressing $\alpha$-$SMA^+$ thus proving that all combinations of these three cell markers were present. In our study the number of $Y^+/\alpha$-$SMA^+$ cells was higher than the number of $CD34^+/\alpha$-$SMA^+$ cells suggesting that other BM derived cell populations such as mesenchymal stem cells or fibrocytes were also possibly contributing to the myofibroblasts population. A rodent model of carbon tetrachloride/ Thioacetamide induced liver cirrhosis, has demonstrated that BM stem cells contribute significantly to hepatic stellate cell and myofibroblast populations and originate from both mesenchymal and HSC populations (Russo et al. 2006). Furthermore our results are compatible with Abe et al who describes a circulating population of fibrocytes in human peripheral blood that *in vitro* could be induced to express $\alpha$-$SMA^+$ and it has been documented that fibrocytes express haematopoietic markers $CD34^+/CD45^+$, myeloid markers and collagen (Abe et al. 2001;Bucala et al. 1994). From this study we have shown that BM derived stem cells can become myofibroblasts or $CD34^+$ expressing cells in the alcohol injured liver. Furthermore, it is also possible that a population of $CD34^+$ cells within the liver was contributing to the recipient derived myofibroblast population in alcohol liver injury ***(see figure 40)***. The exact origins of these $CD34^+/\alpha$-$SMA^+$ cells is uncertain although the possibility exists that they could be derived from circuilating mesenchymal or HSC stem cells or alternatively from an endogenous hepatic source.

### 6.3.4 Hepatic parenchymal cell proliferation

BM stem cells are known to play a role in stimulating endogenous hepatocyte proliferation in a paracrine fashion as evidenced by their contribution in rodent and human studies (Yannaki et al. 2005; Gaia et al. 2006). However we failed to demonstrate any increased proliferation of hepatic parenchymal cells in the cross-sex liver grafts with alcohol liver injury, suggesting that this was not a significant mechanism by

which endogenously mobilised stem cells promote liver repair in alcohol liver injury. It is possible that BM stem cell stimulation of endogenous hepatocyte proliferation occurs in other forms of liver injury that was not examined in this study. Furthermore it is possible that myofibroblasts and other mesenchymal-type cells in alcohol induced liver injury may generate morphogens or growth factors that facilitate liver repair via alternative mechanisms other than hepatocyte proliferation however this possibility was not examined in the current study.

## 6.4 Inflammatory cytokines and chemokines regulating mobilisation and hepatic recruitment of HSCs

### 6.4.1 SDF-1/CXCR4

We demonstrated that AH patients with raised circulating $CD34^+$ levels have significantly increased serum SDF-1 levels as compared to controls in non time course blood samples. Currently there is no published evidence to suggest that Pentoxifylline has any effect on SDF-1 levels although such an influence could be addressed by taking serial blood samples from AH patients both pre and post Pentoxifylline treatment to see any temporal or quantitative effect it may have on serum SDF-1 levels. Furthermore, these results could be compared with a time course evaluation of serum SDF-1 levels from AH patients not treated with Pentoxifylline. Several studies have demonstrated increased circulating plasma levels of SDF-1 in autoimmune and viral diseases, in conjunction with increased expression of SDF-1 in the parenchyma of rejecting liver transplants and viral/autoimmune liver diseases (Goddard et al. 2001; Terada et al. 2003) suggesting that liver injury may by the expression of SDF-1, produce a concentration gradient, which in turn facilitates the recruitment of inflammatory cells and HSCs from the BM into the circulation. We failed to demonstrate an increase in circulating serum levels of SDF-1 in the paracetamol and chronic liver injury groups although these were non time course blood samples and our results may be influenced

by the timing of our samples in relation to the kinetics of the process. Murine studies from our laboratory have demonstrated that both circulating $CD34^+$ and serum SDF-1 levels are elevated in cocaine induced periportal liver injury with an associated increase in the plasma to BM ratio of SDF-1. This would suggest that reversal of the BM/peripheral blood SDF-1 gradient with an increase in plasma SDF-1 is associated with stem cell mobilisation (Gilchrist et al. 2007). The chemokine assay has allowed us to demonstrate elevated SDF-1 concentrations in serum of AH patients, although future studies would need to measure bone marrow SDF-1 levels to determine if an SDF-1 gradient is established between the BM and peripheral circulation which in turn facilitates HSCs mobilisation in alcohol induced liver injury. Hepatic SDF-1 expression has been reported in a variety of conditions such as liver allograft rejection (Goddard et al. 2001), viral and autoimmune liver diseases (Kollet et al. 2003; Terada et al. 2003). It is however unclear if this expression is an attempt to recruit inflammatory cells or HSCs towards the damaged organ, or if it is indeed entirely unrelated. In our study SDF-1 immunohistochemical staining was reduced in the AH liver biopsy sections when compared to the normal controls and SDF-1 expression was confined to the biliary epithelium. Only rodent studies with HOC regeneration models have demonstrated increased hepatic SDF-1 protein up-regulation (Hatch et al. 2002), whilst non-oval cell regeneration liver injury models did not produce SDF-1 protein. We would have expected SDF-1 expression to be increased in the liver parenchyma of AH patients however this was not the case. Possible explanations for this observation are: 1) Our sample size for both groups were small and the normal control group contained pre transplant donor biopsies and specimens with no clinical history which may not have been the most appropriate and could have affected our results, 2) The liver biopsy specimens provided only one snapshot interpretation and may not be the most appropriate time point temporally and 3) The possibility that AH liver injury inhibits both hepatocyte and endogenous hepatic progenitor cell regeneration resulting in reduced hepatic SDF-1 protein expression.

The AH, NC, paracetamol and chronic liver injury groups all had similar HSC CXCR4 receptor profiles suggesting that chemokine receptor expression was not significantly altered in the mobilisation process of $CD34^+$ stem cells in liver injury. The functionality of the CXCR4 receptors was however not tested and it is possible that CXCR4 receptor function on circulating $CD34^+$ cells could have been altered despite any changes seen in receptor expression. CXCR4 receptor function could be assessed in future experiments employing methods such as Transwell chemotaxis assays to SDF-1 concentration gradients as described by Kollet (Kollet et al. 2003) or measuring changes in intracellular calcium in circulating $CD34^+$ cells in response to SDF-1 sensitisation.

### 6.4.2 MMP-9

Serum MMP-9 levels were elevated in non time course blood samples from AH patients (whose circulating $CD34^+$counts were raised) whilst they were not significantly elevated in the serum of either the acute paracetamol or chronic liver injury groups. This suggests that MMP-9 expression in alcohol induced liver injury was a specific response and a previous study by Watanabe et al has demonstrated that in mice with anti-Fas antibody (Jo2) induced acute hepatitis, MMP-9 expression in the circulation was elevated and accompanied by the recruitment of HSCs from the BM into the circulation (Watanabe, Haruyama, & Akaike 2003). It is possible that the observed rise in serum MMP-9 levels in AH patients influences HSC mobilisation however MMP-9 is also known to play various important roles in tissue inflammation, and organ remodelling (Birkedal-Hansen et al. 1993;Visse & Nagase 2003) all of which may offer an alternative explanation for this observed rise. Previous studies have demonstrated elevated serum and plasma MMP-9 levels in various types of acute and chronic liver injuries although this observation may reflect the role MMP-9 has on tissue inflammation, tumour growth and organ remodelling. It is however also possible that the timing of blood samples the severity of the liver injury groups studied and the

use of Pentoxifylline treatment in some AH patients may have influenced the results too. Currently there is no published evidence to suggest that Pentoxifylline has any effect on MMP-9 levels although such an influence could be addressed by taking serial blood samples from AH patients both pre and post Pentoxifylline treatment and compared with a time course evaluation of serum MMP-9 levels from AH patients not treated with Pentoxifylline to see any temporal or quantitative effect.There was no apparent difference in the intensity or amount of MMP-9 staining in liver tissue between the AH and NC groups, suggesting that MMP-9 may be produced by other cell types such as circuilating neutrophils, endothelial cells or connective tissue cells rather than parenchymal cells in the liver and be responsible for the elevated serum MMP-9 levels observed in AH patients.

### 6.4.3 G-CSF

Serum G-CSF levels were elevated in AH patients whose circulating $CD34^+$ cell counts were significantly increased, suggesting that G-CSF may have an effect on mobilising circulating $CD34^+$. Furthermore, Lemoli at al has demonstrated that in OLT patients with ischaemic reperfusion injury a significant increase in circulating $CD34^+$ cells was associated with elevated serum G-CSF levels (Lemoli et al. 2006). We did not detect elevated levels of serum G-CSF or $CD34^+$ levels in either the acute paracetamol or chronic liver injury groups suggesting that G-CSF enhanced mobilisation of $CD34^+$ cells was a specific response to alcohol induced liver injury as compared to acute paracetamol injury and the other forms of chronic liver injuries studies. Factors such as timing of samples, degree of severity of the other forms of liver injury studied and the effect Pentoxifylline may have on G-CSF levels would need to taken into consideration although currently there is no published evidence to suggest that Pentoxifylline has any effect on G-CSF levels.

Murine liver injury studies have suggested that G-CSF promotes endogenous repair and proliferation of host hepatocytes in the injured liver. We did not have liver biopsies available from the AH patient group with elevated G-CSF and $CD34^+$ levels to test this hypothesis directly although indirectly we observed that the cross-sex liver grafts with alcohol induced liver injury did not demonstrate an increase in the proliferation of hepatic parenchymal cells. It may be possible that G-CSF promotes myofibroblasts and other mesenchymal-type cells in alcohol induced liver injury to generate morphogens or other growth factors that facilitate liver repair however this possibility was not examined in our current study. A recent human clinical study in which G-CSF was administered to AH patients to induce HSC mobilisation did however demonstrate an in increases hepatocyte growth factor and a proliferation of hepatic progenitor cells in liver biopsies (Spahr et al. 2008). Whether these changes translated to a clinical improvement was not assessed and perhaps larger human clinical trials may clarify whether G-CSF can clinically and biochemically facilitate liver repair.

### 6.4.4 Chemokines IL-8, MIG, IP-10, RANTES, MCP-1 and NE

Serum IL-8, IP-10 and MIG levels were significantly raised in all types of liver injury and may reflect a non specific inflammatory chemokine response to different types of liver injury. These chemokines play important roles in recruiting inflammatory cells including T-lymphocytes and neutrophils, and it is likely that the high levels are involved in the recruitment of inflammatory cell infiltrate rather than specifically CD34 cells. The chemokines MIG and IP-10 mediate their effect through chemokine receptor CXCR3 and the chemokine receptors expression profiles on HSCs were not significantly altered in all the liver injury groups and NC groups studied. As with CXCR4, we did not specifically study for CXCR3 receptor function. Serum RANTES and MCP-1 levels also demonstrated non specific responses to liver injury. Although serum NE levels were significantly elevated in AH patients, this increase did not correlate with serum MMP-9 or $CD34^+$ levels suggesting that it was a marker for

increased neutrophil activity only rather than demonstrating a role in regulating $CD34^+$ stem cells. Factors such as timing of samples, degree of severity of the liver injury models studied and the effect Pentoxifylline may have on these chemokine levels make it difficult to draw any firm conclusions on the influence these mediators may have in HSC physiology and alcohol liver injury. Liver injury is a complex process, with a complex biochemical soup of mediators released at any one time, many of which have overlapping effects (Simpson et al, 2003). In order to understand the true effects a chemokine may be having, it is necessary to look at the whole picture and assess functional gradients. That is, to measure the levels in the bone marrow, temporal and quantitative measurements in serum as well as to obtain quantitative and special characterisation of its levels within the liver.

## 6.5 Future studies

The data presented in this thesis has been extracted from three different cohorts of patients and in the human setting it is difficult to ascertain if HSCs are modified or primed in alcohol liver injury to contribute to liver repair or fibrosis. Murine studies would be better placed to answer some of these key questions and attention could then be directed as to why such cells do not result in adequate liver repair in clinical practice. HSCs may well contribute to the reparative response, but the degree of stem cell mobilisation may be inadequate, necessitating agents such as G-CSF. Furthermore, endogenous stem cells may also be damaged in the alcohol liver injury process so autologous sources of stem cells may be required for liver repair. In non-transplant patients the presence of stem cell-derived parenchymal cells or fibroblasts cannot be determined making assessment of stem cell contribution difficult. Stem cells ($CD34^+$) from alcohol liver injury and other types of acute and chronic liver injury patients could be infused into pre-irradiated NOD-SCID mice and compared with similar cells obtained from non-liver injury patients receiving G-CSF. Similarly $CD34^+$ cells from alcohol liver injury and non-liver injury patients receiving G-CSF could be infused into

pre-irradiated NOD-SCID mice with ethanol induced toxicity. Immunohistological analysis of murine livers could then be performed using human specific markers for hepatocyte, biliary epithelium, myofibroblast, stem cells and endothelial cells markers. Further evidence of human haematopoietic and hepatic parenchymal/myofibroblast differentiation could be determined with established techniques of immunofluorescence and fluorescent *in situ* hybridisation (FISH) for mouse and human DNA.Data has shown that pre-treatment of circulating human $CD34^+$ cells with anti-CXCR4 antibody enhances their migration and repopulating potential into the bone marrow of NOD-SCID mice (Plett et al. 2002).Investigate whether pre-treating stem cells (from alcohol liver injury and GSCF stimulated non-liver injury patents) with anti-CXCR4 prior to infusion will enhance their migration and hepatocytic differentiation in the NOD-SCID mice livers would be of clinical relevance as it could demonstrate a potential method of manipulating and priming stem cells for liver repair.

Human clinical trials have utilised G-CSF mobilised hematopoietic stem cells to study the short term effects on liver regeneration and liver function. Future human clinical randomised controlled trials in AH patients treated with G-CSF could assess the long-term effects on clinical outcome and liver regeneration. Furthermore such studies could utilise bone marrow, serum and liver tissue samples to assess the changes in the concentration of SDF-1, MMP-9, TNF-α, IL-8 and HGF in response to G-CSF and correlate these changes with the degree of mobilisation and liver regeneration.

## 6.6 Conclusion

In conclusion we have made the observation that human BM derived haematopoietic stem cells are mobilised into the peripheral circulation and recruited into the liver during alcohol induced liver injury. Whilst there was an expansion of HSCs and hepatic progenitor cells within the liver there was no evidence of increased hepatocyte proliferation. We demonstrated that in alcohol induced liver injury there is a marked increase in the proportion of $Y^+$ hepatic myofibroblasts suggesting that BM derived recipient cells were contributing to the liver myofibroblast population. Furthermore, a proportion of the myofibroblasts co-stained for CD34 suggesting that a population of $CD34^+$ cells within the liver were contributing to the hepatic myofibroblast population. Serum SDF-1, MMP-9 and G-CSF levels were elevated in AH and could play a role in regulating the mobilisation of $CD34^+$ stem cells in alcohol induced liver injury.

Figure 40

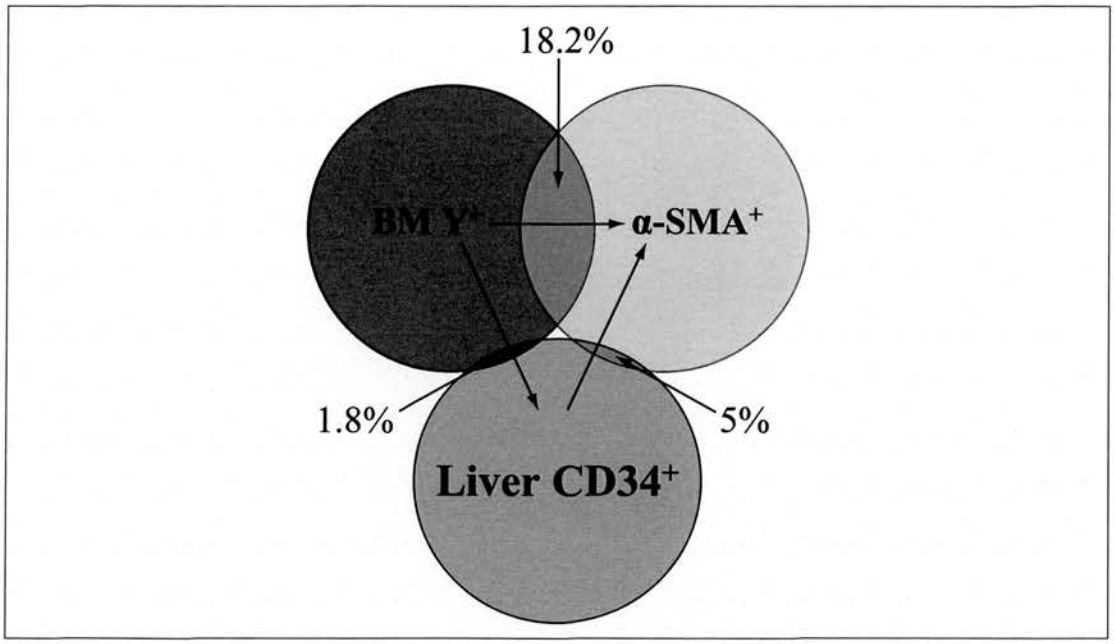


**Figure 40: BM derived stem cells contribute to CD34$^+$ and α-SMA$^+$ liver population in alcohol liver injury.**

BM derived stem cells can become myofibroblasts or CD34$^+$ expressing cells in the alcohol injured liver. Hepatic CD34$^+$ cells also contribute to the hepatic myofibroblast population.

# Epilogue

Current evidence suggests that HSC contribution to hepatocyte lineages is limited and that BM stem cells may contribute to fibrogenesis within the liver. We have demonstrated that HSC as a subset of the BM stem cell compartment have the ability to contribute to pro-fibrotic cells in response to alcohol induced liver injury. It is possible that BM stem cells may support liver repair through other mechanisms such as production of growth factors, fibrosis resolution or even neoangiogenesis although this is yet to be fully established. A more comprehensive understanding is needed of the effect HSCs may have on the diseased liver and there is a theoretical concern that BM stem cell therapy may promote fibrogenesis in cirrhotic livers or even have the potential for malignant transformation. Future studies would need to establish a better understanding of the factors regulating HSC homing, subsequent engraftment into the liver and finally differentiation into various liver cell lines before the potential therapeutic manipulation of HSCs to treat liver disease is to be fully realised.

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# Appendix

## Publications resulting from studies presented in this thesis

1. Dalakas E, Newsome PN, Harrison DJ, Plevris JN. Review article. **Hematopoietic stem cell trafficking in liver injury.** FASEB Journal. 2005 Aug; 19 (10):1225-31.

## Manuscripts in the process of peer review

1. Dalakas E, Newsome PN, Boyle S, Brown R, Pryde A, McCall S, Hayes PC, Bickmore WA, Harrison DJ, Plevris JN. **Bone marrow derived stem cells contribute to liver fibrosis in alcohol induced liver injury.**

# Abstracts and meetings

1. Dalakas E, Newsome PN, Qing L, Brown R, McCall S, Hayes PC, Harrison DJ, Plevris JN. **Liver Injury 'Naturally' Mobilises Bone Marrow Haematopoietic Stem Cells.**

   Oral presentation at *The Scottish Society of Gastroenterology Meeting* (June 2003).

2. Newsome PN, Dalakas E, Qing L, McCall S, Hayes PC, Harrison DJ, Plevris JN. **Liver Injury 'Naturally' Mobilises Pluripotent Bone Marrow Haematopoietic Stem Cells.**

   Oral presentation at *The European Association for the Study of the Liver Monothematic Conference* (September 2003).

3. Dalakas E, Newsome PN, Qing L, Brown R, McCall S, Hayes PC, Harrison DJ, Plevris JN. **Liver Injury 'Naturally' Mobilises Bone Marrow Haematopoietic Stem Cells.**

   Oral presentation at *The British Association for Study of the Liver (BASL) Annual Meeting* (September 2003).

4. Dalakas E, Newsome PN, Qing L, Brown R, McCall S, Hayes PC, Harrison DJ, Plevris JN. **Mobilisation of Pluripotent Haematopoietic Stem Cells occurs in Alcoholic Hepatitis and is associated with an Improved Clinical Outcome.**

   Poster presentation at *The American Association for the Study of Liver Diseases Annual Meeting* (October 2003).

5. Dalakas E, Newsome PN, Hart M, O'Neil K, Lee P, Payne C, Hayes PC, Harrison DJ, Plevris JN. **The Cytokines, MMP-9 and SDF-1, Play a Central Role in Regulating the Mobilisation of $CD34^+$ Stem Cells in Human Liver Injury.**

   Poster presentation at *The American Association for the Study of Liver Diseases (AASLD) Annual Meeting* (October 2004).

6. Dalakas E, Newsome PN, Hart M, O'Neil K, Lee P, Payne C, Hayes PC, Harrison DJ, Plevris JN. **The Acute Inflammatory Response in Alcohol Liver Injury Plays a Role in the Mobilisation of $CD34^+$ Stem Cells.**

   Oral presentation at *The Scottish Society of Gastroenterology Meeting* (November 2004).

7. Dalakas E, Newsome PN, Hart M, O'Neil K, Lee P, Payne C, Hayes PC, Harrison DJ, Plevris JN. **SDF-1, MMP-9 and G-CSF Regulates Human $CD34^+$ Stem Cells in Clinical Liver Injury.**

   Poster presentation at *The Scottish Stem Cell Network Meeting* (March 2005).

8. Gilchrist ES, Newsome PN, Dalakas E, Payne C, Lee P, Cowan P, Pryde A, Hayes PC, Harrision DJ, Plevris JN. **Acetaminophen-induced Fulminant Hepatic Failure results in an SDF-1 mediated 'all or nothing' haematopoietic stem cell response.**

   Poster presentation at the *British Association for the Study of the Liver (BASL) Meeting* (September 2005).

   Poster presentation at *The American Association for the Study of Liver Diseases (AASLD) Annual Meeting* (November 2005).

9. Dalakas E, Newsome PN, Boyle S, Pryde A, Bickmore WA, Hayes PC, Harrison DJ, Plevris JN. **Haematopoietic stem cells are recruited into the liver and contribute to hepatic myofibroblast differentiation in alcohol induced liver injury.**

   Oral presentation at *The Scottish Society of Physicians 49th Annual Scientific Meeting* (September 2007).

10. Dalakas E, Newsome PN, Boyle S, Pryde A, Bickmore WA, Hayes PC, Harrison DJ, Plevris JN. **Alcohol Induced Liver Injury Mobilises Haematopoietic Stem Cells that Contribute to Hepatic Fibrosis.**

    Poster presentation at *The European Association For The Study of The Liver Annual Meeting* (April 2008) and *The British Society of Gastroenterology Annual Meeting* (March 2008).

11. Dalakas E, Newsome PN, Boyle S, Pryde A, Bickmore WA, Hayes PC, Harrison DJ, Plevris JN. **Alcohol Induced Liver Injury Mobilises Bone Marrow Derived Stem Cells that Contribute to Liver Fibrosis.**

    *Oral presentation at TheUnited European Gastroenterology Week Meeting* (October 2008).

# Publications

# Hematopoietic stem cell trafficking in liver injury

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ABSTRACT **Bone marrow (BM) hematopoietic stem cells (HSCs) have been shown to facilitate regeneration in multiple nonhematopoietic tissues by either generating epithelial cells or altering the inflammatory response. Depending on injury type, the predominant mechanism of epithelial lineage regeneration occurs by spontaneous cell fusion or transdifferentiation. Irrespective of the mechanism, mobilization from the BM is a prerequisite. Mechanisms by which HSCs mobilize into damaged organs are currently under scrutiny. Murine and human studies have shown that the chemokine SDF-1 and its receptor CXCR4 participate in the mobilization of HSCs from BM and in the migration of HSCs to injured liver. SDF-1 is a potent HSC chemoattractant and is produced by the liver. Production is increased during liver injury leading to increased HSC migration to the liver, a finding diminished by neutralizing anti-CXCR4 antibodies. Additional factors have been implicated in the control of hepatic migration of HSCs such as IL-8, hepatocyte growth factor, and MMP-9. Matriceal remodeling is an essential component in HSC engraftment, and MMP-9 expression is increased in liver injury. This review focuses on the complex interaction of chemokines, adhesion molecules, and extracellular matrix factors required for successful migration and engraftment of HSCs into the liver.—Dalakas, E., Newsome, P. N., Harrison, D. J., Plevris, J. N. Hematopoietic stem cell trafficking in liver injury. *FASEB J.* 19, 1225–1231 (2005)**

*Key Words: migration · engraftment · SDF-1 · MMP-9*

BONE MARROW (BM) hematopoietic stem cells (HSCs) have long been known to possess the unique capacity for self-renewal and differentiation into hematopoietic and mesenchymal cell lineages (1). That this plasticity extended to nonhematopoietic lineages such as hepatic oval cells, hepatocytes, cholangiocytes (1–3), skeletal muscle cells (4), neurons (5), epithelial cells of the lung, GI tract, and skin (6) is a relatively new observation, and has raised hopes that such cells could in the future be used for the regeneration and reconstitution of damaged organ tissue. This process of epithelial lineage regeneration appears to occur via a mechanism of spontaneous cell fusion or transdifferentiation. Emerging data in the field of cardiac regeneration suggest that incoming stem cells can also contribute to tissue repair by promoting neoangiogenesis and minimizing cardiomyocyte apoptosis (7). Whatever the underlying mechanism by which the HSCs participate in tissue regeneration, it will still require the presence of HSCs to mobilize from the BM and reach their target organ. The aim of this review is to summarize current available information addressing the aspects of HSC mobilization and trafficking in response to liver injury.

## ADULT STEM CELLS AND LIVER REGENERATION

While the liver is a mitotically quiescent organ in adult humans and animals (8), hepatocytes have a remarkable capacity to meet the replacement demands during cellular loss (9, 10). However, when either chronic/extensive damage is inflicted on the liver or when hepatocyte proliferation is inhibited, a facultative cellular compartment of hepatic oval cells (HOCs), located within the smallest branches of the intrahepatic biliary tree is activated and leads to liver repair (10, 11). More recently, several groups have demonstrated that BM-derived HSCs may contribute to liver repair (1–3, 12–14). The contribution of HSCs to liver repair has varied, but is generally related to the presence and severity of liver injury. Thus, the restitutive response of the liver to different injuries has been proposed to include three levels of proliferating cells: *1)* the hepatocyte, *2)* the endogenous ductular progenitor cell or HOC, and *3)* a pluripotent stem cell derived from circulating BM cells (9).

Controversy has recently arisen as to whether HSCs contribute to the hepatocyte lineage in liver injury via transdifferentiation alone or by adopting the phenotype of hepatocytes after spontaneous cell fusion (15). Recent reports in favor of the fusion hypothesis have demonstrated that adult cells can adopt the phenotype of other cell lines by fusing with embryonal stem cells (16, 17) as well as BM-derived hepatocytes generated by in vivo cell fusion (18). In support of transdifferentiation, several groups have demonstrated that HSCs can differentiate into hepatocytes (19, 20) and pancreatic endocrine cells (21) without any evidence of cell fusion. The mechanism of HSC hepatic regeneration

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doi: 10.1096/fj.04-2604rev

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remains unresolved; clearly, any future stem cell research will have to distinguish HSC transdifferentiation from fusion events. Whatever the mechanism of hepatic regeneration is, the trafficking of HSCs to the liver may play an important component of the reparative process in liver injury.

The contribution of HSCs to hepatocyte lineages in rodents and humans remains a controversial area with data both supporting (1–3, 13, 14, 22) and rebutting (23–25) findings. This may in part reflect the types of cells used, the injury models used, and the methods used to detect stem cell progeny. Nevertheless a therapeutic role of HSCs in liver injury has been described in rodents (13, 26), albeit with varying contributions of transdifferentiation and fusion. In other models, particularly in humans, the contribution that HSCs make to liver repair by transdifferentiation is lower, on the order of 0.011–20% (2, 3, 6, 27–29). To improve on this level of contribution will require greater understanding of the mechanisms by which stem cells mobilize from the BM and home to injured organs. There remains a pressing need for further studies to confirm or refute the claims that stem cells can lead to improved liver repair and hence survival in either a rodent or human setting.

Murine and human studies have shown that the chemokine stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, are involved in recruiting inflammatory cells into injured livers as well as inducing proliferation of endogenous HOCs (30, 31). SDF-1/CXCR4 interactions participate in the mobilization of HSCs from BM and have been implicated in the migration of human HSCs to the liver during injury (32, 33). Other factors have been implicated in the regulation of hepatic migration of HSCs, including interleukin-8 (IL-8), hepatocyte growth factor (HGF), and matrix metalloproteinases (MMPs).

## ADULT STEM CELL MOBILIZATION AND RECRUITMENT IN LIVER INJURY

Human studies have demonstrated increased levels of circulating HSCs in response to a systemic injury such as acute sickle cell crisis and surgical trauma (34, 35). A recent study by De Silvestro et al. demonstrated that peripheral blood HSC levels were elevated after extensive liver resection (36). Our group has demonstrated that in patients with alcoholic hepatitis there is an increase in circulating HSCs when compared with normal controls (37). The extent to which these peripheral blood HSCs are mobilized into the circulation of patients with liver injury and contribute to liver repair remains uncertain and is under investigation.

### Release of HSC from the bone marrow

In the adult BM, the release of HSCs into the peripheral circulation is regulated in part by the CXC chemokine SDF-1 and its receptor CXCR4 (32, 33, 38, 39). SDF-1 is a potent chemoattractant for HSCs and is produced by various BM stromal cell types and epithelial cells in a broad range of normal tissues, including the liver (40–46) (see **Table 1**). It plays a major role in the homing, migration, proliferation, differentiation, and survival of many cell types including human and murine hematopoietic stem/progenitor cells (32, 33, 38, 39, 47–50). Knockout mice deficient in SDF-1 exhibit disturbed hematopoiesis and knockout mice deficient in the CXCR4 receptor die in utero (51, 52), underlining their importance.

SDF-1 is highly conserved between mice and humans (53, 54), mediating its effect through the CXCR4 receptor that is expressed on $CD34^+$ HSCs, mononuclear leukocytes, and a variety of stromal cells (53). CXCR4 is a G-protein-coupled, 7-transmembrane receptor and is the only known receptor for SDF-1 (55). The interaction between SDF-1 and CXCR4 has been demonstrated to trigger multiple intracellular signals, including calcium mobilization and phosphorylation of adhesion components such as extracellular signal-regulated kinases 1 and 2 (ERK-1 and -2), proline-rich tyrosine kinase 2 (Pyk-2), focal adhesion kinase (FAK), and protein kinase C (PKC) (56, 57) In the adult BM, release of HSCs into the peripheral circulation is controlled in part by a concentration gradient of SDF-1 established within the BM microenvironment (39, 58, 59). Reduction of BM SDF-1 levels has been shown to result in release of HSC into the peripheral circulation, an effect mediated partly by granulocyte colony-stimulating factor (G-CSF), which induces the release and proliferation of neutrophil proteases such as elastase, cathepsin G, and MMPs (33). Increased expression of SDF-1 in the peripheral circulation facilitates further mobilization of HSCs down a concentration gradient (60).

Several reports demonstrate increased circulating plasma levels of SDF-1 in autoimmune and viral diseases, in conjunction with increased expression of SDF-1 in the parenchyma of rejecting liver transplants and viral/autoimmune liver diseases (31, 61). These observations have been reported in murine liver injury models (12, 30), suggesting that liver injury may, by the

TABLE 1. *SDF-1 expression in normal human tissue*

| Tissue type | Cell line SDF-1 expression |
|---|---|
| Bone marrow | Stromal cell lines |
| Tonsil | Epithelial cells in tonsillar crypt |
| Spleen | Reticular cells |
| Fetal liver | Mesothelial cells, biliary epithelium, ductal plate |
| Adult liver | Biliary epithelium |
| Lung | Interstitial cells |
| Cardiac | Cardiac myocytes |
| Brain | Glial cells, cortical neuronal cells, astrocytes |
| Muscle | Skeletal myocytes |
| Skin | Epithelial cells of sweat glands, endothelial cells, pericytes, dendritic cells |
| Thymus | Stromal cells, medullary cells, epithelial cells |

expression of SDF-1, produce a concentration gradient between liver and BM, which in turn facilitates the recruitment of inflammatory cells and HSCs from the BM into the circulation and then into the liver (12, 31) (see **Fig. 1*A–C***).

The mechanism by which SDF-1 influences HSC mobilization is unclear, although it is thought to involve specific changes to the adhesion of progenitor cells to the BM microenvironment via the modulation of adhesion molecules such as the integrin-dependent very late antigen-4 (VLA-4) (62). In vitro there is an increased trans-endothelial migration of human progenitor cells toward a gradient of SDF-1 (48, 63), and SDF-1 has been shown to promote the survival of circulating $CD34^+$ HSCs by counteracting apoptosis via the activation of the phosphotidyl inositol 3 kinase (PI3-K)/Akt pathway (64).

It has been speculated that the release of proteolytic enzymes and chemokines from injured liver into the circulation could also facilitate mobilization and recruitment of HSCs (12). Studies with G-CSF have revealed neutrophil proteolytic enzymes such as elastase, cathepsin G, and MMPs, including MMP-2 and MMP-9, result in the proteolytic degradation of SDF-1 in the BM, thus facilitating the release of stem cells (33, 65). MMPs degrade extracellular matrix proteins and are known to play important roles in tissue inflammation, tumor growth, and organ remodeling (66, 67). MMPs are secreted as zymogens (pro-MMPs) that are activated by a variety of proteinases and inhibited by tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin (66). In humans, MMP-9 is produced in a wide variety of cells types such as neutrophils, progenitor cells, endothelial cells, fibroblasts, connective tissue cells, tumor cells, and parenchymal cells, including the liver (66, 68). Human and animal studies have demonstrated that MMP-9 promotes the release of progenitor cells from the BM into the circulation by *1)* inducing the release of soluble kit-ligand (sKitL) from BM stromal cells, which accelerates the proliferation and migration of HSCs, *2)* cleaving the interaction of adhesion molecules VLA-4/vascular cell adhesion molecule-1 (VCAM-1) between stromal cells and HSCs in the BM, and *3)* enhancing the SDF-1 induced migration potential of HSCs across the subendothelial basement membrane (38, 69–71). In addition, MMP-9-induced recruitment of HSCs may occur via other mechanisms such as the shedding of membrane-bound stem cell factor (SCF) and the secretion of MMP-9 by progenitor cells in response to SDF-1 stimulation (70, 71). MMP-9 has been demonstrated to have an active involvement in liver remodeling in cirrhosis and inflammation as well as regulating hepatocyte regeneration after partial hepatectomy (72–74).

Human studies have demonstrated elevated serum and plasma MMP-9 levels in various types of liver injury including acute allograft rejection (75), ischemic reperfusion injury (76, 77), chronic viral hepatitis (78, 79), and alcoholic liver cirrhosis (80), suggesting there is a correlation between disease severity/progression and MMP-9 expression. In these studies, 70–80% of the serum and plasma MMP-9 measured, appeared in the active complex form and could be detected in serum samples from as early as 30 min and >1 wk after an acute injury process. In chronic liver diseases such as alcoholic cirrhosis, persistently elevated plasma activities of MMP-9 have been demonstrated, suggesting its expression reflects a process of ongoing extracellular matrix remodeling (80). Carbon tetrachloride (CCl4) -induced liver injury studies in rats and NOD/SCID mice (in which bone marrow cells were seen to transdifferentiate into hepatocytes) demonstrated an increased expression and activation of MMP-9 in the liver, suggesting that this factor could potentially be involved

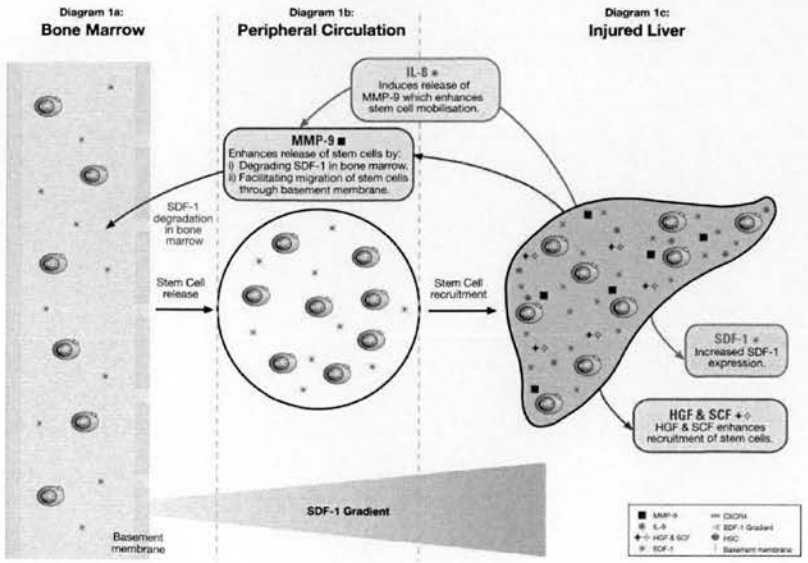


**Figure 1.** *A)* Release of HSCs from bone marrow into peripheral circulation mediated via SDF-1 concentration gradient. *B)* Release of HSCs into peripheral circulation enhanced via MMP-9 and IL-8. *C)* Recruitment of HSCs into injured liver mediated via SDF-1, HGF, and SCF.

in the stress-induced recruitment of HSCs from the BM to the injured liver (12, 81). A recent study by Hanumegowda et al. (82) has demonstrated an increased activation of MMP-9 in the livers of rats with monocrotaline-induced liver injury (which inhibits hepatocyte proliferation and promotes an HOC response). This increase in MMP-9 activity was produced from either the endothelial cells or from an activation or influx of inflammatory cells into the injured hepatic parenchyma (82). In a study by Watanabe et al, mice were injected with anti-Fas antibody (Jo2) to induce an acute hepatitis, demonstrating that MMP-9 expression in the circulation was elevated and accompanied by the recruitment of HSCs from the BM into the circulation (83).

Interactions between MMP-9 and other chemokines such as IL-8 have been demonstrated in mobilization studies whereby MMP-9 is rapidly induced in neutrophils after exposure to IL-8 and resulting in the release of HSCs into the peripheral circulation (84–87) (see Fig. 1*B*). Elevated IL-8 levels have been demonstrated in the circulation and hepatic parenchyma of many human liver conditions including alcoholic hepatitis, viral hepatitis, chronic alcoholic liver disease, and acute graft-vs.-host disease after liver transplantation (88–92). Thus IL-8, a known neutrophil chemoattractant in liver disease, also has the potential to induce the release of HSCs into the peripheral circulation via an indirect mechanism requiring the activation of circulating neutrophils and the release of MMP-9 (86).

### Hepatic recruitment of HSCs in liver injury (Fig. 1C)

Kollet et al. have recently demonstrated the key role that SDF-1/CXCR4-mediated signaling plays in the migration of human progenitors to the murine liver. Neutralization of the CXCR4 receptor with an anti-CXCR4 antibody significantly inhibited the homing of human cord blood or mobilized peripheral blood $CD34^+$ stem cells to the liver of irradiated NOD/SCID mice (12). Furthermore, injection of human SDF-1 into the murine liver parenchyma further enhanced the hepatic migration of human stem cells. SDF-1 expression has been reported in a variety of liver and nonliver conditions such as liver allograft rejection (61), viral and autoimmune liver diseases (12, 31), ischemic brain injury (93), myocardial infarction (94), inflammatory skin conditions (44), and BM injury induced by total body irradiation or chemotherapy (95). It is unclear whether this expression is an attempt to recruit inflammatory cells or HSCs toward the damaged organ or is indeed entirely unrelated.

SDF-1 expression in rejecting liver transplants and viral/autoimmune liver diseases was seen to be confined to the biliary epithelium and other nonparenchymal cells, thus promoting the retention of $CXCR4^+$ lymphocytes and possibly HSCs in the portal tracts (12, 31, 61). Hatch et al. (30) were able to demonstrate that SDF-1 protein was up-regulated in the membrane fraction of the whole liver lysates. Notably, however, this was only the case in animals that had undergone HOC regeneration models [partial hepatectomy (PH) and 2-acetylaminofluorene (2-AAF) or 2-AAF and CCl4]. Animals that had undergone non-oval cell regeneration models of PH, CCl4 alone, and 2-AAF alone did not produce SDF-1 protein. Immunohistochemistry on the oval cell regeneration model liver sections revealed increased expression of SDF-1 in the hepatocytes adjacent to the proliferating oval cells and positive CXCR4 staining on these oval cells. These data argue for the defined production of SDF-1 in forms of liver injury that may be attempting to recruit HSCs to the reparative process.

The cytokine HGF, which is produced in the nonparenchymal perisinusoidal cells of the liver and induces hepatocyte proliferation, may also be involved in the migration and differentiation of HSC into the injured liver (12, 96). Increased expression of HGF has been demonstrated in CCl4-induced liver injury and in rodent HOC regeneration models, suggesting it is involved in stem cell proliferation, migration, and differentiation (22, 97). Kollet et al. recently demonstrated that after liver injury, levels of HGF were increased and contributed to the recruitment of human $CD34^+$ stem cells to the injured liver (12) by increasing the motility of human progenitors and in synergy with SCF potentiated both CXCR4- and SDF-1-induced directional migration.

## CONCLUSIONS

Many concepts regarding stem cell migration and plasticity come from studies of multipotent hematopoietic stem cells and the molecular pathways of hematopoiesis (98). There is now increasing evidence to suggest that liver injury induces the expression and secretion of signaling mediators such as SDF-1, IL-8, MMPs, HGF, and SCF, which facilitate the homing and engraftment of HSCs to the liver (12, 30, 31). Factors regulating long-term engraftment and differentiation of HSCs into hepatocytes are yet to be defined, although chemokines, adhesion molecules, and extracellular matrix factors would appear to have an important role to play. A better understanding of the factors regulating HSC homing, subsequent engraftment into the liver, and finally differentiation into hepatocytes are essential if the potential therapeutic manipulation of HSCs to treat liver disease is to be realized. FJ

E.D. was supported by a University of Edinburgh Research Fellowship Award. Figure 1 designed by Wendy Richardson from The University of Edinburgh Medical Illustration Unit.

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*Received for publication July 21, 2004.*
*Accepted for publication February 24, 2005.*

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